The dna26-1 mutation of Saccharomyces cerevisiae

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The *dna26-1* Mutation of *Saccharomyces cerevisiae*

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ABSTRACT

The *dna26-1* mutation was originally identified during a screen for temperature sensitive mutations causing a rapid arrest of DNA synthesis under restrictive conditions. Initial studies on the original *dna26-1* mutant, including extensive complementation analysis, order of function mapping and physiological characterization, indicated that the *dna26-1* mutation was a novel *cdc* mutation with an early execution point in the cell cycle and a putative Class I START mutation. The aim of the present study was to further characterize the physiological effects of the *dna26-1* mutation and to clone the wild type *DNA26* gene.

A temperature sensitive mutant, TDE/16A, was derived from the original *dna26-1* mutant JL448 by three rounds of backcrossing to a wild type strain, screening segregants for the *cdc* phenotype. Physiological analysis of TDE/16A under restrictive conditions revealed that, i) the G1-arrest phenotype of the mutant was leaky, ii) the mutant displayed a degree of conjugational competence and resistance to elevated temperature that was intermediate to that characteristic of Class I and Class II START mutants and iii) the mutant displayed a rapid and severe decrease in the apparent rate of protein synthesis characteristic of a Class II START mutant. These results were unexpected as the original *dna26-1* mutant had been clearly shown to cause a G1-arrest phenotype characterisitic of Class I START mutations. An influence of genetic background on the *dna26-1* mutation was therefore revealed.

A yeast genomic fragment capable of functionally complementing the *dna26-1* mutation in TDE/16A was isolated from a gene library in

several independent transformation events. Tn5-mediated transposon mutagenesis of the cloned fragment located the *dna26-1*-suppressing activity to a 2.9kb region within a 3.75kb *Cla*I fragment. This *Cla*I fragment was capable of suppressing the *dna26-1* mutant phenotype both after sub-cloning to a low copy centromeric plasmid and after integration into the TDE/16A genome as a single extra copy.

Linkage mapping carried out to establish the site of integration of the cloned fragment proved inconclusive. A strict interpretation of the data obtained was that the cloned fragment had integrated at a genomic locus that was linked to, but nevertheless extragenic to the the *dna26-1* locus. However it was uncertain whether the data from the tetrad analysis were being complicated by the appearance of unexpected temperature sensitive mutations. The same problem had been encountered during the strain construction of TDE/16A preventing the *dna26-1* mutant phenotype from being conclusively attributed to a single chromosomal mutation. The evidence for linkage between the site of integration of the clone and the *dna26-1* locus was however used to map the physical location of the *DNA26* gene. Using the cloned fragment to probe a Southern blot of *S. cerevisiae* chromosomes the *DNA26* locus was mapped to chromosome XV.

The restriction map of the clone was compared with those of previously isolated genes known to be capable of mutating to give a START-arrest phenotype. The map of the cloned sequence was found to be almost identical to that of the cloned *PRT1* gene. Despite previous evidence that the *prt1* mutation was capable of complementing the *dna26-1* mutation, the combined data compiled from the physiological and molecular analysis of the *dna26-1* mutation in TDE/16A suggested that the *dna26-1* mutation is an allele of *PRT1*.

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CONTENTS

CHAPTER 1 : GENERAL INTRODUCTION

1.1 YEAST AS A SYSTEM FOR STUDY OF THE CELL CYCLE

The mitotic cell cycle coordinates the process of asexual reproduction. It comprises a series of discontinuous events that occur against a background of continuous mass accumulation via protein and ribonucleic acid (RNA) synthesis and energy metabolism. The events of the cell cycle ensure the accurate segregation of duplicated genetic material and cellular components between two cells in a process of cell division.

Several properties of the yeast *Saccharomyces cerevisiae* make it an appropriate organism for the study of the cell cycle. *S. cerevisiae* is a simple eukaryote that displays elements of chromosome replication and segregation, macromolecular synthesis and cell structure that are essentially homologous to those of higher eukaryotic cells (Hartwell, 1974). The genomic organisation of *S. cerevisiae* is similar to that of higher eukaryotic organisms in that the genes are randomly distributed over the whole of the genome with little evidence of clustering of functionally related genes (Mortimer & Schild, 1985). The process of cell division by budding in this yeast provides a morphological marker for cell cycle progression. The emergence of a new bud occurs soon after cell cycle initiation and increases in size as the cycle proceeds towards the production of a mother and daughter cell. *S. cerevisiae* is also a non-pathogenic yeast that does not mate or exchange DNA with any bacterial or fungal pathogen and that harbours no known transmissible virus (Botstein *et al.*, 1979).

The greatest utility of *S. cerevisiae* in the study of the cell cycle is the tractability of its genetic systems. It has a haploid and

diploid stage to its life cycle (Figure 1.1) and the same gene-controlled events occur during the mitotic division of *S. cerevisiae* in the haplophase and the diplophase (Hartwell *et al.*, 1974). This allows the isolation of novel recessive mutations in the haploid cell and their further analysis by complementation in the diploid cell. Genetic analysis of *S. cerevisiae* is further facilitated by its clonability, ease of handling and storage and its capacity to grow on a defined medium andundergo replica-plating (Mortimer & Hawthorne, 1969). The size and physical robustness of the cells also makes them amenable to visualization and micromanipulation under the light microscope. Sophisticated protocols for the application of recombinant DNA technology and molecular biological techniques to the study of yeast genes have also been developed (Struhl, 1983). The ability to produce synchronously dividing populations of cells is an important requirement for the study of the cell cycle (see Wheals, 1987). Techniques for the induction of synchrony in yeast cell populations include the treatment of cells with peptide mating pheromones and cell cycle inhibitors and nutrient starvation. Populations can also be fractionated with regard to the stage in the cell cycle by size selection in an elutriation rotor. This selection synchronisation of a cell population can be achieved without removal of cells from the culture medium thus causing a minimum perturbation of cellular processes.

The above traits are also characteristic of the yeast *Schizosaccharomyces pombe*, although this yeast divides by a process of fission rather than budding. A large number of cell cycle genes from *S. cerevisiae* and *S. pombe* have been studied. These analyses have revealed that many of the genes involved in cell cycle control

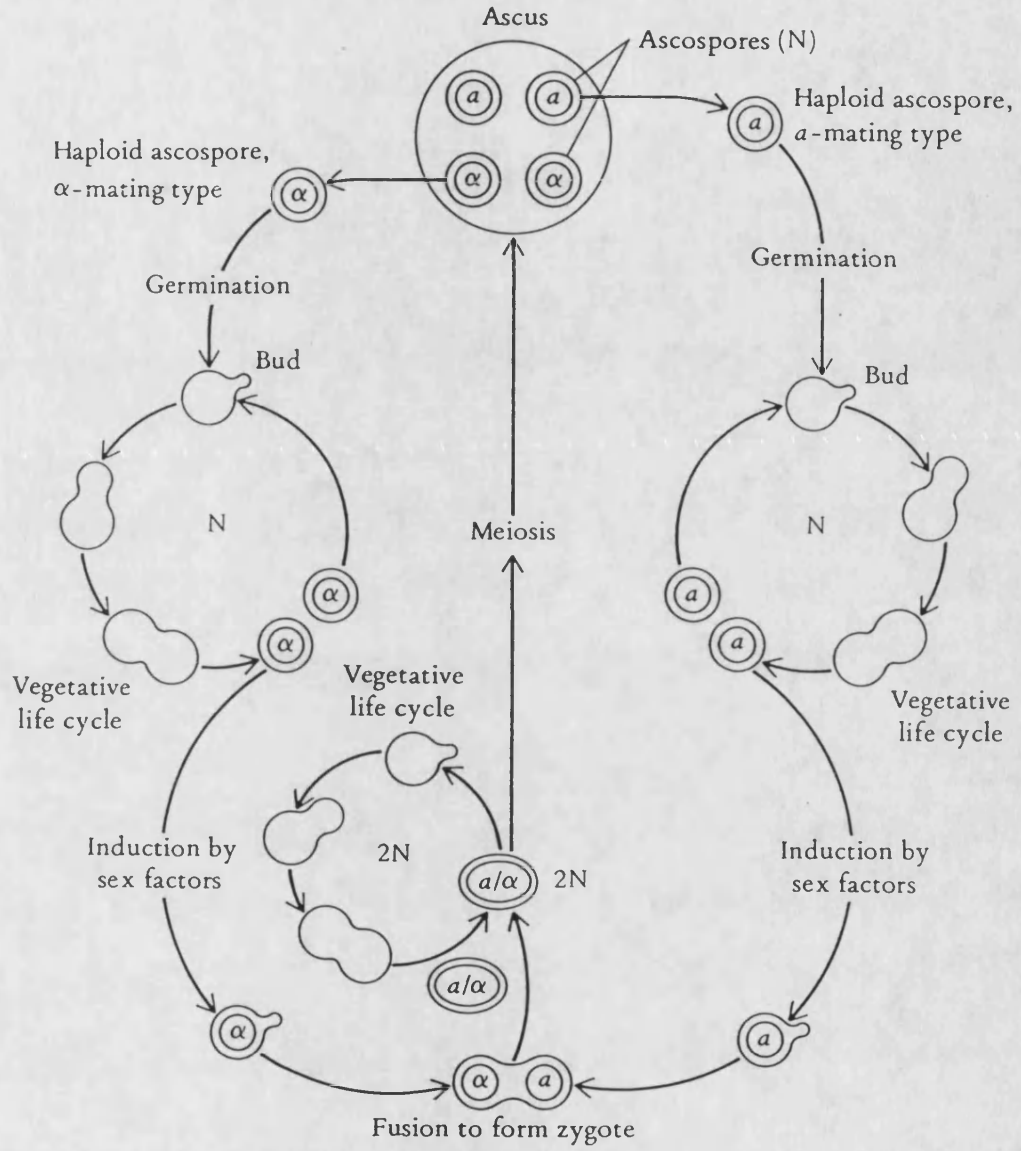


FIGURE 1.1 Life Cycle of *Saccharomyces cerevisiae*

(From Russell, 1986).

in yeast possess a structural and functional homology to mammalian oncogenes (for reviews see Wheals, 1985; Nurse, 1990). Mutation or inappropriate expression of mammalian protooncogenes or the insertion of viral oncogenes into the eukaryotic genome can cause neoplastic transformation of mammalian cells. This leads to uncontrolled cell division, tumour formation and cancer. The apparent evolutionary conservation of cell cycle control mechanisms permits the genetic information gained from the study of proliferation control in yeast to be correlated with related physiological studies carried out on higher eukaryotic cells (Murray & Kirschner, 1989). The understanding of the mechanisms of oncogenic transformation should lead to the development of more specific and effective cancer therapies.

1.2 THE MITOTIC CELL CYCLE OF *SACCHAROMYCES CEREVISIAE*

As for higher eukaryotic cells, the cell cycle of *S. cerevisiae* can be divided into four stages. A period of genomic DNA synthesis (S-phase) and a period of nuclear division (M-phase) are each preceded by a gap phase (G1 and G2 respectively). The gap phases are considered to be periods of preparation for the synthesis and then the segregation of the genetic material.

A temporal series of morphological, cytological and biochemical landmarks have been discerned during the *S. cerevisiae* cell cycle (Figure 1.2). At the start of the cycle in G1 the cells are unbudded with a single undeveloped spindle pole body embedded in the nuclear membrane. In late G1 the spindle pole body, which is the microtubule organising centre of the cell, develops a satellite structure. The satellite is the precursor of a duplicate spindle pole body and this

FIGURE 1.2

Major Landmarks of the *S. cerevisiae* Cell Cycle

Duration of events not to scale.

Abbreviations

SPBSF : spindle pole body satellite formation

SPBD : spindle pole body duplication

CRF : chitin ring formation

MRF : microfilament ring formation

BE : bud emergence

iDS : initiation of chromosomal DNA synthesis

DS : chromosomal DNA synthesis

SPBS : spindle pole body separation

NM : nuclear migration

mND : medial stage of nuclear division

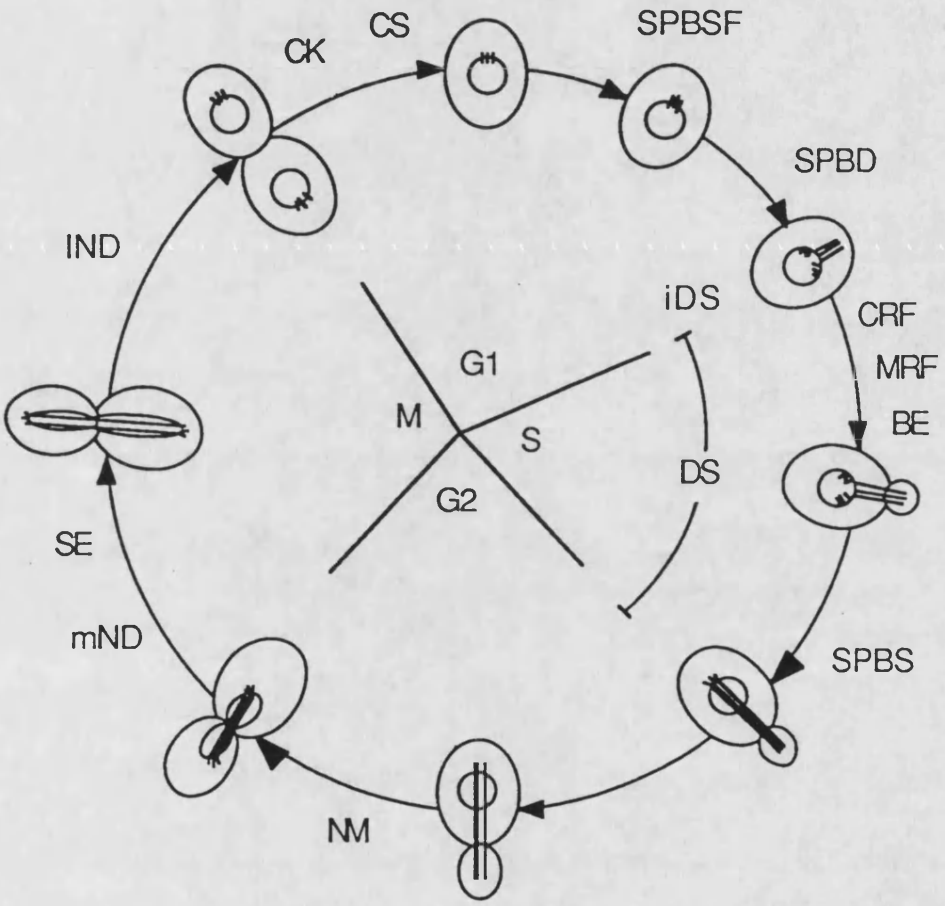
SE : spindle elongation

lND : late stage of nuclear division.

CK : cytokinesis

CS : cell separation

(From Pringle & Hartwell, 1981)


CK
CS
SPBSF
SPBD
CRF
MRF
BE
SPBS
NM
mND
SE
IND
iDS
DS
G1
S
G2
M

duplication heralds the emergence of a new bud. A ring of chitin is first laid down at a site in the cell wall that is in close proximity to the duplicated spindle pole body. Rings of filamentous material are then observed to form at the isthmus joining the mother cell and emerging bud (see Wheals, 1987). The initiation of DNA synthesis occurs at about the same time as bud emergence (Hartwell, 1976) and bud enlargement continues throughout S-phase. By the end of S-phase the spindle pole bodies have separated to opposite poles of the nucleus and are connected by intranuclear microtubules. This early formation of a spindle structure before the onset of M-phase is unusual and may contribute to the short and ill-defined G2 phase in the *S. cerevisiae* cell cycle. At the end of G2 the nucleus migrates to the bud isthmus and the spindle then elongates with the spindle pole bodies moving to opposite poles of the mother cell and bud. M-phase proceeds with the segregation of nuclear material within a complete nuclear membrane. Prophase condensation of the yeast chromosomes is not visible in *S. cerevisiae* which may be due to their small size (Pringle & Hartwell, 1981). Karyokinesis is achieved as deposition of new membrane material at the narrowest point of the bud isthmus causes the nuclei to pinch apart. Cytokinesis occurs in early G1 followed by the deposition of new wall material at the site of cell cleavage leading to cell separation.

In order to replicate itself successfully a cell must be able to reproducibly coordinate the stage-specific functions of the cell cycle such as those outlined above. Furthermore it must be able to integrate these functions with the continuous processes of cellular growth and metabolism. A genetic analysis to determine how the cell cycle is controlled in *S. cerevisiae* was originally initiated by the

isolation of 148 conditional lethal, temperature sensitive mutants that were defective in a particular stage-specific function of the cell cycle (Hartwell *et al.*, 1970; 1973; 1974; Culotti & Hartwell, 1971). These mutations were assigned to 32 complementation groups defining single nuclear genes involved in cell cycle control. Each mutation was termed conditionally lethal in that it permitted the continued proliferation of cells at the permissive temperature of 23°C, but after a shift to the restrictive temperature of 36°C the mutant cells arrested at a particular stage in the cell cycle. A characteristic of *cdc* mutant strains is therefore that the cells arrest at the restrictive temperature as a morphologically homogenous population, with the final morphology depending on the particular *cdc* mutation present.

The initial effect of a *cdc* mutation is said to be on the primary defect event (Pringle & Hartwell, 1981). This may be due to a defect in the structure and/or function of the mutant gene product or in its synthesis. The consequences of the mutant defect may not however be manifested until later in the cell cycle. The first event that can be discerned to be affected by the primary defect is therefore defined as the diagnostic landmark. Cell cycle events that are not dependent on the primary defect event do however continue at the restrictive temperature as do the continuous processes of growth. The terminal phenotype of a *cdc* mutant population is therefore often distinct from any normal stage of the cell cycle (Pringle, 1978). Each *cdc* mutation also has an execution point within the cell cycle. Execution point has been defined as the time in the cell cycle after which a shift to restrictive conditions can no longer prevent the mutant cell from successfully completing the current cell cycle. The

corollary of this is that a tight conditional *cdc* mutant that is before the execution point at the time of the temperature shift will display a first cycle arrest. Conversely, a mutant cell that has progressed beyond the execution point at the time of the shift will complete the current cell cycle and arrest with the appropriate terminal phenotype in the next.

Three approaches have been taken to investigate the temporal order of cell cycle events and their functional inter-relatedness; execution point analysis, double mutant analysis and order-of-function mapping (reviewed by Pringle, 1978). Execution point analysis has been used to temporally map the order of gene-controlled events giving information about the time in the cell cycle when a gene product is synthesized or when it completes its essential function. The technique involves shifting an asynchronous *cdc* mutant population to the restrictive temperature and determining the fraction of cells that are capable of completing the current cell cycle. This can be carried out by monitoring cell number increase in liquid culture or by time-lapse cinematography of cells on solid medium. The disadvantage of this technique is that it requires the use of tight conditional labile mutations to generate a convincing temporal map. Furthermore the execution point of a *cdc* mutation may also vary with genetic background generating variation in the temporal map.

Functional sequence mapping is based on the hypothesis that the events of the cell cycle are ordered into a series of dependent and parallel pathways. Two gene functions may be ordered in a dependent pathway with function-B being dependent on the prior completion of function-A or *vice versa*. Alternatively function-A and function-B may be interdependent, with neither function being

performed in the absence of the other. Finally, function-A and -B may be independent of each other (Jarvik & Botstein, 1973; Hereford & Hartwell, 1974).

In double mutant analysis the conditional phenotype of two mutants carrying a different *cdc* mutation is compared with that of a double mutant carrying both mutations. This technique is appropriate for the study of temperature sensitive mutations as the restrictive condition for both mutations can be applied simultaneously by the shift of an asynchronous culture to 36°C. The method requires that both mutations cause the same spectrum of blocked and permitted events at the restrictive temperature, but cause distinct terminal phenotypes. This firstly establishes that their primary defect events are not interdependent. Secondly, if the primary defect events occur in a dependent sequence, then the double mutant will display the terminal phenotype resulting from blockage at the earliest primary defect event. If however the two mutations operate in independent pathways of the cell cycle, then the phenotype of the double mutant should be distinct from that of either single mutant. The disadvantage of this technique is that it requires the use of mutations that cause a clear block in a normal cell cycle event rather than promoting an abnormal activity. The method also requires that the execution points of the two single mutations be close together. This ensures that only an insignificant fraction of the double mutant population is likely to be at a stage in the cell cycle between the execution points of the two mutations at the time of the temperature shift. A further drawback of this technique is that a unique terminal phenotype resulting from the independent function of the two single mutations in the same cell cannot always be distinguished from the

phenotype of both single mutants.

The other widely used method of functional sequence mapping is reciprocal shift analysis. This technique involves the consecutive application of two reversible cell cycle blocks (for example caused by a temperature sensitive *cdc* mutation and a cell cycle specific inhibitor). The method involves two experiments (Table 1.1). In the first, the cells are accumulated at a stage in the cell cycle that is sensitive to blocking agent A. The cells are held under this restrictive condition for a length of time during which an untreated population would have completed the step sensitive to blocking agent B. The cells are then released from the block caused by agent A and immediately arrested with agent B. From this experiment it can be determined whether the cell cycle step sensitive to agent B was completed during the initial arrest with agent A. If so, the cells are observed to complete one cell cycle during the second incubation before arresting at the step that is sensitive to agent B. The second experiment is the reciprocal of the first, with the cells first being blocked with agent B and subsequently with agent A. The method yields information about the order of executability of cell cycle steps and is therefore most accurate when blocking agents are used that cause a tight (but reversible) cell cycle arrest. The disadvantage of this technique is that it may separate stages in the cell cycle that are normally temporally close together. The decay of a vital transient state during the first incubation may therefore indicate a false interdependence between two cell cycle functions.

The number of mutations in *S. cerevisiae* causing a *cdc* phenotype is currently in the region of 100 (see Pringle & Hartwell, 1981; Wheals, 1987). The combination of data from functional sequence

Experimental Protocol		Conclusion
Block at A then release and attempt to block at B	Block at B then release and attempt to block at A	
+	-	A dependent on B
-	+	B dependent on A
+	+	A & B independent of each other
-	-	A & B interdependent

TABLE 1.1 Protocol for Reciprocal Shift Analysis

The above experimental procedure allows the determination of the dependency relationship between two cell cycle events, A and B (see text).

+, cell completes the current cell cycle.

-, cell does not complete the current cell cycle.

(from Wheals, 1987).

mapping experiments involving most of these mutations and a range of cell cycle inhibitors has permitted the construction of a functional sequence map of the *S. cerevisiae* cell cycle (Figure 1.3). These genetic analyses have therefore led to a view of the cell cycle as an ordered programme of events with the gene products that mediate those events being restricted to a prescribed sequence of function. The map consists of a series of dependent and parallel pathways of gene function that are interconnected by points of divergence and convergence. The functional map corresponds well with the temporal map of cell cycle events (see Figure 1.2) although functional mapping data has clearly revealed that a new cell cycle can be initiated without the prior completion of cytokinesis and cell separation.

It is evident from the functional sequence map that the *S. cerevisiae* cell cycle begins with a major control point where a large collection of genes must complete their function before a divergence to the independent pathways of subsequent events. This control point serves to coordinate the discontinuous events of the cell cycle with the continuous processes of growth.

1.3 CELL CYCLE CONTROL AT START

The major rate limiting step in the *S. cerevisiae* cell cycle occurs in G1 and is comparable to the restriction point that is involved in the switch between quiescence and proliferation in animal cells (Pardee, 1974). The completion of this step by yeast cells in G1 marks a commitment to the mitotic cell cycle and has therefore been termed START (Hartwell *et al.*, 1974; Reed, 1980; Wheals, 1987). Arrest of the cell cycle at START can occur in wild type cells as a response to a number of different enviromental circumstances. These

FIGURE 1.3 Functional Sequence Map of *S. cerevisiae cdc* Mutations

The numbers refer to the *cdc* mutation. Diagnostic landmark events are in boxes; dependent events are on consecutive arrows; interdependent events are on the same arrow and independent events are on parallel pathways. The positions of the landmark events across the figure are approximately to a correct temporal scale for the normal cell cycle.

Abbreviations are as for Figure 1.2 except for :

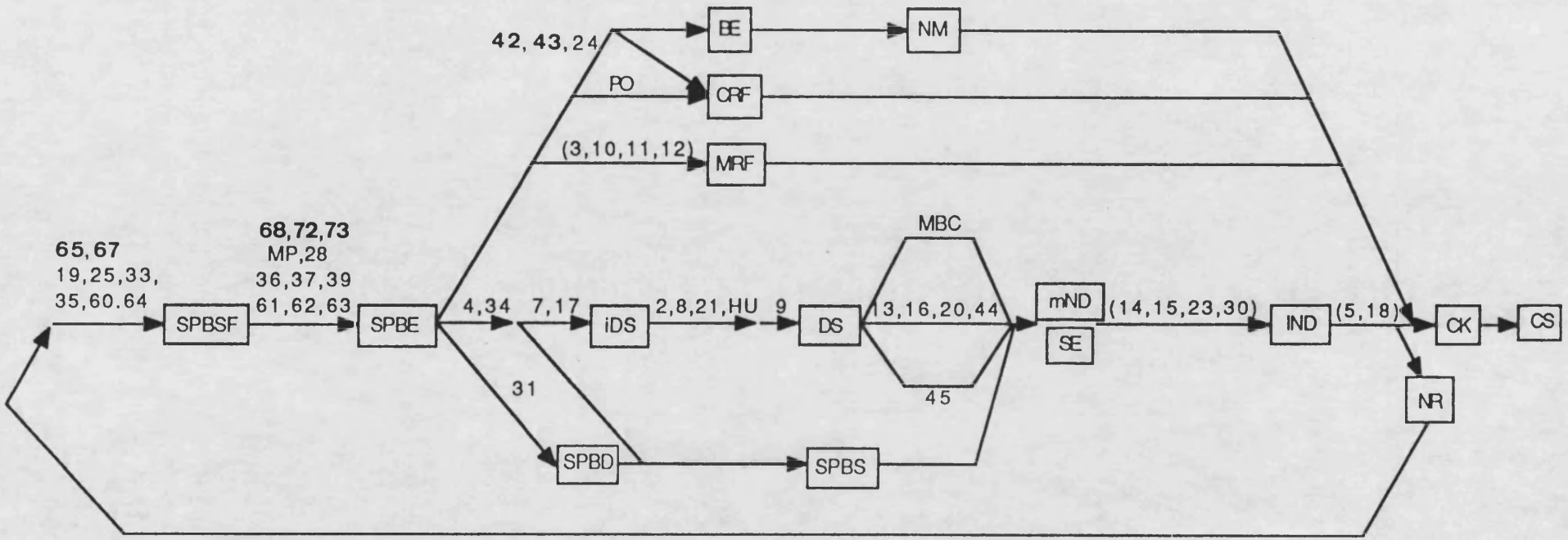
MP : mating pheromone PO : polyoxin D

HU : Hydroxyurea NR : nuclear reorganization

MBC : methyl benzimidazole-2-ylcarbamate (benomyl)

SPBE: spindle pole body elongation

(From Wheals, 1987)


42, 43, 24
BE
NM
PO
CRF
(3,10,11,12)
MRF
MBC
65,67
19,25,33,
35,60.64
68,72,73
MP,28
36,37,39
61,62,63
SPBSF
SPBE
4,34
7,17
iDS
2,8,21,HU
9
DS
13,16,20,44
mND
SE
(14,15,23,30)
IND
(5,18)
CK
CS
45
31
NR
SPBD
SPBS

include a deprivation of nutrients including carbon and energy sources, NH_4^+, SO_4^+, K^+, PO_4^+, or biotin. The presence of mating pheromone can also cause cell cycle arrest of haploid cells at START. The yeast cell thus integrates many informational inputs at START and mutations that cause arrest of cells at START may do so through discreet signal pathways affecting the cell's choice of developmental programme.

As seen in Figure 1.3, a large number of genes have been identified that function to control the commitment to mitotic proliferation at START. A classification system proposed by Reed (1980) characterizes mutants in these genes on the basis of whether under restrictive conditions they resemble pheromone arrested (Class I) or nutritionally arrested (Class II) cells. Conditional Class I START mutants characteristically maintain rapid growth and the ability to conjugate under restrictive conditions and arrest at START with a spindle pole body bearing a satellite structure (Byers & Goetsch, 1975). Whilst some of the Class I gene products are thought to be directly involved in the mating pheromone signal transduction pathway (see section 1.6) some are thought to be components of the mechanism controlling entry into the mitotic cycle (see section 1.5).

Class I mutants can be further classified by the criterion of mating projection formation (shmoo morphology) under restrictive conditions. Examples of Class I mutations causing shmoo formation include *cdc28, cdc36, cdc37* and *cdc39* (Reed, 1980), *cdc70(gpa1(TS)* - *Jahng et al.*, 1988), *cdc72* and *cdc73* (Reed *et al.*, 1988) and *sra1-1* (Clark & Sprague, 1989). Those that do not cause shmoo formation during START arrest include *cdc61, cdc62* and *cdc63* (Bedard *et al.*, 1981), *cdc68* (Prendergast *et al.*, 1990a) and *mak16* (Wickner, 1988).

A further characteristic of *cdc36* and *cdc39* is that the G1 arrest caused by these mutations in haploid cells is suppressed when the mutations are present in a cell in which the mating pheromone signal transduction pathway is inoperative due to sterility or to heterozygosity at the mating type locus (Connolly *et al.*, 1983; De Barros Lopes *et al.*, 1990). The latter is also a characteristic of the *sra1-1* mutation (Clark & Sprague, 1989). The conditional G1 arrest phenotype is also suppressed in *cdc36* and *cdc39* mutants grown on a medium containing a non-fermentable carbon source (Shuster, 1982b). The *cdc28* and *cdc37* mutations also have a distinctive characteristic. These START mutations cause a karyogamy defect during mating (Dutcher & Hartwell, 1982; 1983).

Class II START mutants have been described as stationary phase-like mutants (Bedard *et al.*, 1981) and are thought to be involved in the growth-related nutrient sensing mechanisms of the START control point. They do not maintain rapid growth during START arrest, nor do they retain the ability to conjugate. Like nutritionally arrested wild type cells they arrest at START with a single, undeveloped spindle pole body (Pringle & Hartwell, 1981) and with a high level of thermotolerance (Plesset *et al.*, 1987). Class II START mutations include *cdc25* and *cdc35* which are involved in a nutrient-responsive signal transduction pathway which controls cellular proliferation via cyclic AMP-dependent protein phosphorylation (see section 1.7). The *cdc19* mutation also causes a Class II START arrest and is thought to be the mutant form of the structural gene for pyruvate kinase (Pringle & Hartwell, 1981). Both the *cdc63* and *cdc33* mutations are thought to be involved in translation (Hanic-Joyce *et al.*, 1987a; Brenner *et al.*, 1988), whilst the *gcd1(tra3)*

and *gcd12* mutations are involved in the general control of amino acid synthesis (Hill & Struhl, 1988; Wolfner *et al.*, 1975; Paddon & Hinnebusch, 1989). A temperature sensitive *ils1-1* mutation encoding a conditionally defective isoleucyl-tRNA synthetase also causes a Class II START arrest. Other Class II START mutations include *cdc60* and *cdc64* (Bedard *et al.*, 1981) and *cdc67* (Prendergast *et al.*, 1990a).

All the above START mutants function at about the same point in G1 and no gene functions have been identified upstream of this point. The Class I mutations appear to function downstream of the Class II mutations at START. However, definitive reciprocal shift experiments carried out in order to functionally map the point of Class I and Class II START arrest in relation to the execution point of alpha-factor have failed to unequivocably distinguish the points of arrest (see Iida & Yahara, 1984a; Matsumoto *et al.*, 1983). Other mutants have been identified which show a qualitatively different loss of control over START regulation than those above. Mutants in the *ARD1*, *WHI2* or *UBI4* gene fail to arrest in G1 and to display stationary phase characteristics under conditions of nutrient starvation (Whiteway & Szostak, 1985; Saul *et al.*, 1985; Tanaka *et al.*, 1988). These genes therefore appear to encode negative regulators of START.

Depending on enviroment and cell type, a *S. cerevisiae* cell can embark upon one of four developmental pathways from START (Figure 1.4). Under conditions of nutrient starvation cells pass into a stationary phase state called G0 (see Bedard *et al.*, 1982). There has been debate over whether G0 is a unique developmental state or whether the physiological characteristics of stationary phase merely reflect the stress responses leading to cell cycle arrest (for

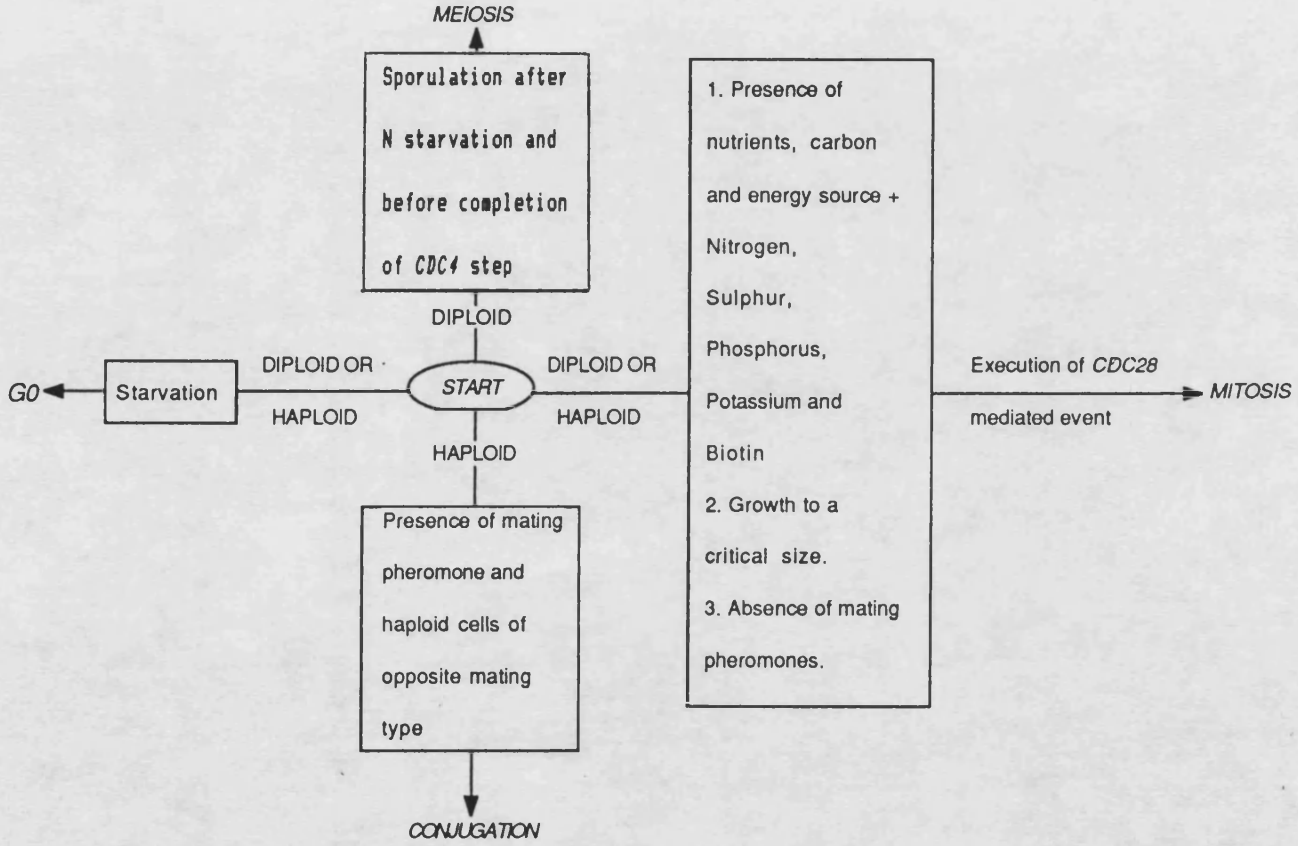


FIGURE 1.4 Possible Developmental Pathways from START

Cells may develop in one of four ways from START, depending on the enviroment and cell type. To read, begin at START and follow any of the possible developmental pathways. (Adapted from Hartwell, 1974 and Wheals, 1987).

example see Iida & Yahara, 1984a; Bedard *et al.*, 1981). The former view has however been supported recently by the isolation of a mutant strain that is conditionally defective only for the resumption of proliferation from stationary phase and which has no effect during exponential growth (Drebot *et al.*, 1987; 1990).

An alternative developmental programme that can only be executed from START is that of conjugation (Reid & Hartwell, 1977; and reviewed by Cross *et al.*, 1988). Haploid yeast cells produce peptide mating pheromones that are specific to the cell's mating type (Bucking-Throm *et al.*, 1973; Wilkinson & Pringle, 1974). Reciprocal exchange of mating pheromones by cells of opposite mating type leads to synchronization of their cell cycles by arresting them at START (Hereford & Hartwell, 1974). A process of conjugation then occurs during which the two cells fuse at the cytoplasmic and nuclear level, usually resulting in the formation of a diploid cell.

Under conditions of nitrogen starvation on a non-fermentable carbon source, diploid cells may embark upon meiotic cell division from START, or from the interval between START and the execution point of *cdc4* (Hirschberg & Simchen, 1977). Most of the *CDC* genes that operate in the mitotic cell cycle have also been shown to be required for meiosis (Simchen, 1974; Shuster & Byers, 1989). The four sets of segregated chromatids resulting from the reductional and equational divisions of meiosis are encapsulated by wall material forming four spores. These are retained within an ascal sac and under appropriate nutritional conditions the spores may germinate and reinitiate mitotic division.

Under favourable conditions of nutrient availability and in the

absence of mating pheromone, cells may reinitiate a mitotic cell cycle. However, in order to do this they must meet one further criterion. In order to execute START the cells must attain a minimum cell size and this size requirement for START is accentuated in *S. cerevisiae* by the asymmetric mode of division.

1.4 ASYMMETRIC DIVISION AND MODELS FOR SIZE CONTROL AT START

During the process of budding in *S. cerevisiae* the mother cell almost always gives rise to a daughter cell that is smaller than itself at division (Hartwell & Unger, 1977). This contrasts with the method of division by symmetrical binary fission in most cells. In addition, the cycle time of the daughter cell is longer than that of the larger mother cell. The increased cycle time results from the longer period spent in the unbudded (G1) phase of the cell cycle with the S-G2-M period of mothers and daughters remaining equivalent in duration. These observations have led to the hypothesis that cell cycle control is mediated by a critical size requirement for cell cycle initiation operating in G1 (Hartwell & Unger, 1977; Lord & Wheals, 1980).

The model of cell size control is consistent with the observation that growth is rate limiting for cell division rather than *vice versa* (Johnston *et al.*, 1977a; see also Singer & Johnston, 1985) and that the rate limiting step occurs at or before the cell cycle step mediated by the *CDC28* gene product (Johnston *et al.*, 1977a). The critical size model also explains the requirement of small, nutrient starved, G1-arrested cells for a period of growth before traversing START (Johnston *et al.*, 1977a).

Even though during the exponential growth of *S. cerevisiae* almost all

the new material is deposited in the growing bud, the daughter cell is almost always smaller than the mother cell at division. At all but maximal growth rates, the daughter cell requires a period of growth to attain the critical size for START. Conversely, the mother cell can usually initiate a new cell cycle almost immediately after cell division. The doubling time of a population of growing cells is therefore a composite of the different mother and daughter generation times and the fraction of daughter cells in a population increases as the growth rate decreases (Carter and Jagadish, 1978). The critical size model can explain each of these observations if it is considered that the mother is already at the critical cell size at division whilst the daughter cell is smaller and requires a period of growth before reinitiating a new cell cycle (see Figure 1.5).

Additional observations have however been made that are not predicted by the simple size control model including the following :-

i) The critical size for START is indirectly proportional to the population doubling time (Lorincz & Carter, 1979). Experiments on different carbon sources have revealed that cells growing at a low growth rate initiate the cell cycle at a smaller size than those growing at a faster rate, although further evidence has indicated that cell size is modulated by the level of catabolite derepression rather than growth rate itself (Mountain & Sudbery, 1990; Baroni *et al.*, 1989). Furthermore, once the population doubling time has decreased to a certain value the critical size becomes independent of growth rate so that a minimum size for START is reached.

ii) Mother cells may increase in volume by up to 23% in each successive generation with most of the increase occurring in the

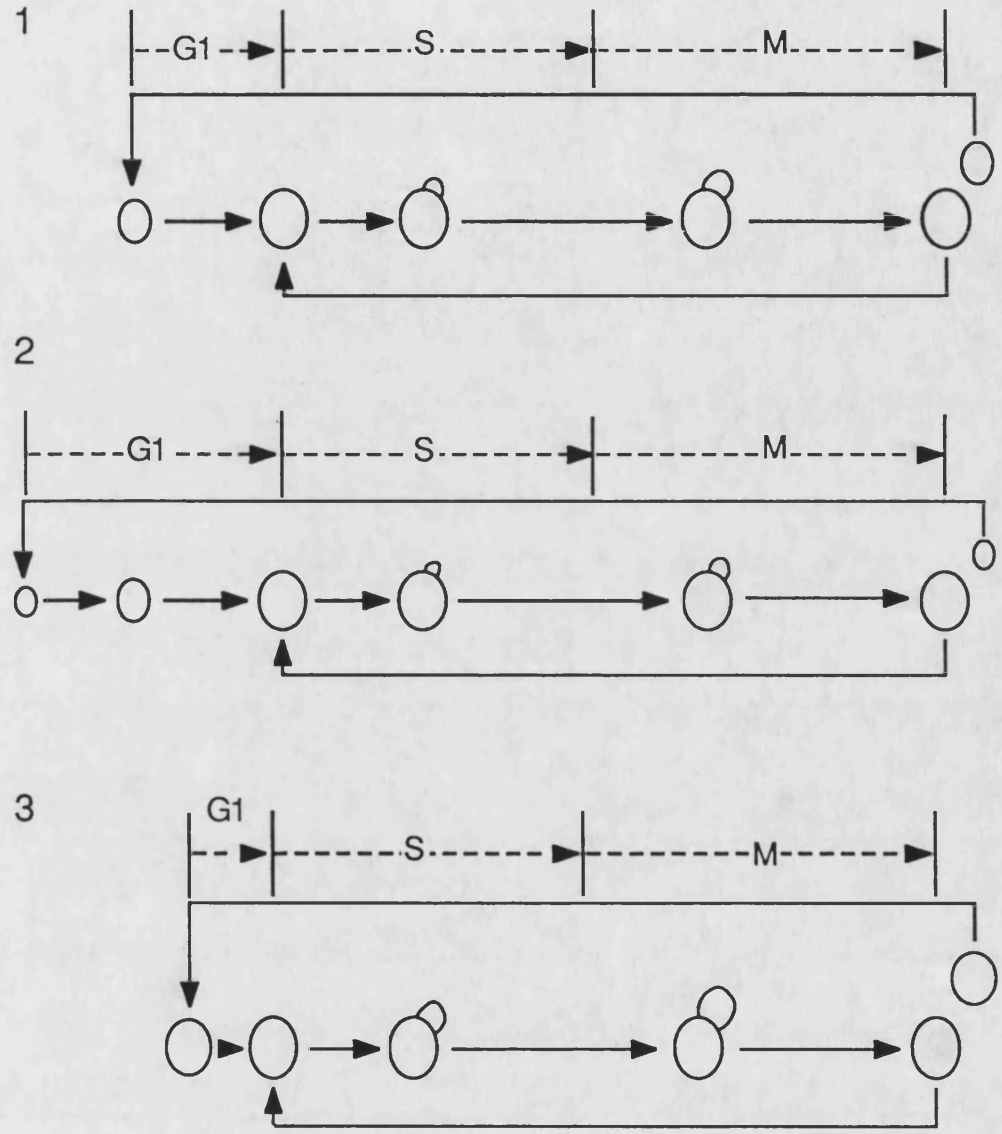


FIGURE 1.5 Effect of Growth Rate on the Length of G1 in Daughter Cells

The rate at which cells grow affects the length of time the daughter cells spend in G1. If the S and M periods are fixed, the amount of mass a daughter acquires is dependent on how fast cells are growing. With 1 as balanced growth, at a slower growth rate, 2, the daughter is smaller at cell separation and so must spend longer in G1. At faster growth rates, 3, G1 is reduced as mother and daughter are closer in size at cell separation.

unbudded interval of the cycle (Hartwell & Unger, 1977).

iii) Although the budded interval of the cell cycle is relatively constant, it does show an increase in duration at slow growth rates (Wheals, 1987).

Additional observation of the cycle time and critical size of individual cells in an exponentially growing population has revealed a marked variability in both parameters for both mother and daughter cells (Wheals, 1982). This has led to the proposal of a model for size control at START that includes both deterministic and probabilistic elements (Wheals,1982; Lord & Wheals, 1983). The sloppy size control model states that the probability of a cell traversing START increases with its increasing size after division. Cells that are born small have a low probability of traversing START whilst those that are born large have a high probability.

Size is a deliberately vague term for the requirement for START. Parameters such as cell volume, cell protein and total cell mass have been monitored to quantify growth. The factor that the cell monitors may in fact be merely a function of one of these cellular parameters. One possibility is that the cell synthesizes an activator protein at a rate that is proportional to the net rate of protein synthesis and which triggers cell cycle initiation at a critical concentration. This hypothetical protein would require two properties i) a high rate of turnover to allow a fast response to changing enviromental conditions and ii) a rate of turnover proportional to the number of genome equivalents in the cell (as diploid cells are approximately twice the size of haploids).

Finally, the observation that cells that are already at the critical

cell size at division can initiate a new cell cycle without an intervening period of growth suggests that the G1 interval is dispensible for successful reproduction. This proposal has been supported by experiments in which the duration of the budded phase of the cell cycle was prolonged by partially inhibiting DNA synthesis (Singer & Johnston, 1981, Johnston & Singer, 1983). Under these conditions the cells had time to grow to the critical size during the budded interval of the cycle and were able to initiate a new cell cycle immediately after nuclear division without the necessity for a pre-START G1 interval of growth. These observations imply that there are no G1-specific events that have to be completed before START and that the period between nuclear division and START is merely a period of growth in preparation for the next round of division. This view of the cell cycle is in accord with the continuum model which desribes the process of cell division as a sequence of events (the DNA-division sequence comprising S, G2 and M) that occur with a variable periodicity that depends on the amount of division potential accumulated between the initiation of successive S-phases (Cooper, 1979; Okuda & Cooper, 1989).

1.5 UNIVERSAL SYSTEMS OF CELL CYCLE REGULATION

The *CDC28* START gene encodes the *S. cerevisiae* homologue of a phylogenetically conserved regulator of the eukaryotic cell cycle. The most extensive genetic analysis of this key cell cycle control element has been carried out in the fission yeast *S. pombe* in which it is encoded by the *cdc2*$^+$ gene. The *cdc2*$^+$ gene functions at the two major control points of the *S. pombe* cell cycle, controlling commitment to the initiation of DNA synthesis at START and entry into

M-phase from G2 (Nurse & Bisset, 1981) and it is the essential role of *cdc2*$^{+}$ in mitosis that has been most intensely studied (Nurse, 1990). The *CDC28* and *cdc2*$^{+}$ genes have been shown to be functionally interchangeable in their heterologous yeast hosts (Beach *et al.*, 1982). The human homologue of the *cdc2*$^{+}$ gene (*CDC2Hs*) has been cloned from a cDNA library by functional complementation of a temperature sensitive allele of the fission yeast homologue (Lee & Nurse, 1987) and has also been shown to functionally complement a deletion of the *CDC28* gene in *S. cerevisiae* (Wittenberg & Reed, 1989). Furthermore a homologue of *cdc2*$^{+}$ has been isolated from a *Pisum sativum* (garden pea) cDNA library using oligonucleotides degenerate with conserved sequences flanking the 16-amino acid PSTAIR motif as primers for the polymerase chain reaction (Feiler & Jacobs, 1990; Lee & Nurse, 1988). From yeasts to humans the *cdc2*$^{+}$ gene encodes a 34kDa serine/threonine protein kinase (p34^{cdc2}) that phosphorylates a conserved S/TPX target motif (where X is a basic amino acid) in as yet poorly defined protein substrates.

The evolutionary conservation of p34^{cdc2} as a central regulator of mitosis has been firmly established by the correlation of genetic data from yeast with biochemical analyses of the cell cycle in amphibian and marine invertebrates (reviewed by Murray & Kirschner, 1989; Maller 1990). Frog and starfish oocytes and eggs have been used extensively for cell cycle studies due to their capacity for synchronous rounds of meiotic and mitotic divisions respectively. A cytoplasmic activity, termed maturation promoting factor (MPF), has been identified in M-phase-arrested unfertilized *Xenopus* (frog) eggs that induces meiotic maturation in immature oocytes even in the presence of protein synthesis inhibitors (Masui & Markert, 1971;

Smith & Ecker, 1971; Gerhart *et al.*, 1984). Entry into M-phase in the frog and starfish systems is also associated with a burst of phosphorylation due to the activation of an M-phase specific (or growth associated) histone H1 kinase (H1K) that is independent of Ca^{++}, diacylglycerol and cyclic nucleotides (Bradbury *et al.*, 1974). The convergence of biochemical and genetic data occurred however with the discovery that histone H1 kinase activity in both *Xenopus* MPF and starfish H1K is due the presence of a 34kDa protein that is immunologically cross-reactive with the $p34^{cdc2}$ kinase from *S. pombe* (Gautier *et al.*, 1988; Dunphy *et al.*, 1988; Arion *et al.*, 1988) leading to the conclusion that MPF and H1K are manifestations of the same activity containing a universal mitotic kinase.

Active MPF contains a component other than the $p34^{cdc2}$ kinase subunit which has been identified as a cyclin (Draetta *et al.*, 1989, Labbé *et al.*, 1989). Cyclins were first identified in studies of cell division in sea urchin embryos (Evans *et al.*, 1983). They are defined as proteins that accumulate during the cell cycle and are rapidly destroyed at anaphase. Cyclins from different organisms are identified at the amino acid sequence level by a central prototypic cyclin box motif of approximately 150 residues. Two types of cyclin, A and B, have been identified with most organisms containing both types (for example see Whitfield *et al.*, 1990). A functional distinction between the two cyclin types has not yet been clearly defined although binding of $p34^{cdc2}$ to a type-A or type-B cyclin is thought to be mutually exclusive (Draetta *et al.*, 1989). The *cdc13*$^{+}$ gene of *S. pombe* encodes a B-type cyclin, $p56^{cdc13}$, which forms a complex with $p34^{cdc2}$ (Solomon *et al.*, 1988; Booher *et al.*, 1989). The cyclin subunit of this complex is required for the activation of

the kinase activity of $p34^{cdc2}$ at the G2/M boundary and subsequently for the formation of the mitotic spindle during mitosis (Booher & Beach, 1988; Moreno *et al.*, 1989). The cyclin may also localise the $p34^{cdc2}$ kinase to the nucleus during M-phase (Booher *et al.*, 1989). The cyclin itself becomes a substrate for the $p34^{cdc2}$ kinase during mitosis and exit into telophase has been shown to be dependent upon cyclin destruction at anaphase.

Activation of the mitotic kinase activity of $p34^{cdc2}$ in *S. pombe* is not only dependent on its association with the $p56^{cdc13}$ cyclin but also on the dephosphorylation of $p34^{cdc2}$ at a specific tyrosine residue located within the ATP-binding site of the protein (Gould & Nurse, 1989). In *S. pombe* this mitotic activation of $p34^{cdc2}$ is regulated by a network of regulatory genes including *cdc25*$^+$, *wee1*$^+$, *mik1*$^+$ and *nim1*$^+$. Mutations in these genes have been identified that can alter the timing of mitosis revealing their regulatory role (and contrasting with the strictly conditional lethal phenotype of most *cdc* genes). The product of *cdc25*$^+$ positively regulates $p34^{cdc2}$ kinase activity and in this respect functions antagonistically to the to the products of the *wee1*$^+$ and *mik1*$^+$ genes (Russell & Nurse, 1986; Russell & Nurse, 1987a; Lundgren *et al.*, 1991). The *nim1*$^+$ gene product positively regulates entry into mitosis by down-regulating *wee1*$^+$ activity and both of these interacting genes display sequence homology to known protein kinases (Russell & Nurse, 1987b). Activation of $p34^{cdc2}$ at the G2/M boundary is also controlled by two type-2 protein phosphatases encoded by the *ppa1*$^+$ and *ppa2*$^+$ genes. Two further type-1 protein phophatases encoded by the *dis2*$^+$ and *sds21*$^+$ genes are thought to regulate later mitotic functions including sister chromatid separation (Kinoshita *et al.*, 1990).

The level of the *cdc25*$^{+}$ gene product, p80^{cdc25}, rises during the cell cycle, peaking towards the end of G2 (Moreno *et al.*, 1990). As well as "fine tuning" the timing of mitosis, p80^{cdc25} controls the dependence of mitosis on the prior completion of S-phase DNA replication (Enoch & Nurse, 1990). This checkpoint control is probably mediated via post-translational modification of p34^{cdc2} (Broek *et al.*, 1991; Krek & Nigg, 1991) and ensures the correct alternation of S-phase chromosome replication and M-phase chromosome segregation. Homologues of *cdc25*$^{+}$ been identified in budding yeast (Russell *et al.*, 1989), *Drosophila* (Edgar & O'Farrell, 1989) and human cells (Sadhu *et al.*, 1990).

One further gene involved in the regulation of p34^{cdc2} activity in *S. pombe*, *suc1*$^{+}$, has been isolated by its ability to suppress a temperature sensitive defect in the *cdc2*$^{+}$ gene at high gene dosage (Hayles *et al.*, 1986a; 1986b). The *suc1*$^{+}$ gene product p13^{suc1} physically interacts with the p34^{cdc2} protein and is thought to mediate exit from mitosis by inactivation of p34^{cdc2} kinase activity (Moreno *et al.*, 1989). Fission yeast p13^{suc1} has been shown to block tyrosine dephosphorylation and activation of the *Xenopus cdc2* kinase (Dunphy & Newport, 1989).

The substrates of the p34^{cdc2} kinase in yeast and higher eukaryotes are yet to be fully elucidated. It is not yet clear whether the *cdc2* kinase acts as a "workhorse" or as a master regulator for cell cycle-dependent phosphorylation. In the first case it would phosphorylate its substrates directly and in the second would phosphorylate secondary regulators that would amplify the initial signal in a phosphorylation cascade. The substrate that is routinely employed for assaying p34^{cdc2} kinase activity is histone H1. Phosphorylation

of this protein may promote mitotic chromosome condensation. Some of the other mitotic substrates of $p34^{cdc2}$ include nucleolar proteins (Peter *et al.*, 1990), intermediate filaments (Chou *et al.*, 1990) and nuclear lamins (Kirschner & Ward, 1990; Heald & McKeon, 1990). Several protooncogenes have also been reported to contain the consensus target sequence for $p34^{cdc2}$ phosphorylation (Maller, 1990; Sturzbecher *et al.*, 1990).

Genetic analysis of the $p34^{cdc2}$ homologue in *S. cerevisiae* encoded by the *CDC28* gene has revealed that the major requirement for *CDC28* gene function occurs at START (Reed, 1980), although this gene function is also required in G2 for entry into mitosis (Piggott *et al.*, 1982; Reed & Wittenberg, 1990). Antibodies raised against the *CDC28* gene product precipitate a 34kDa protein that is capable of phosphorylating an endogenous 40kDa substrate of unknown identity (Reed *et al.*, 1985). $p34^{CDC28}$-dependent phosphorylation of p40 fluctuates during the cell cycle, being maximal at START and decaying after entry into S-phase (Mendenhall *et al.*, 1987; Wittenberg & Reed, 1988). START arrest induced either by the action of mating pheromones, nutritional starvation or mutational inactivation of the *CDC35* gene abolishes $p34^{CDC28}$ kinase activity (Mendenhall *et al.*, 1987). Phosphorylation of p40 at START is dependent upon the assembly of the $p34^{CDC28}$ kinase into a high molecular weight heteromultimeric complex (Wittenberg & Reed, 1988). This complex disassembles after entry into S-phase and also when cells are arrested in G1 in response to mating pheromones or starvation. A partially disassembled complex of intermediate molecular weight extracted from actively growing cells has been shown to possess histone H1 kinase activity and may represent the mitotic form of the

kinase (Wittenberg & Reed, 1988; Reed & Wittenberg, 1990).

A further demonstration of the conservation of cell cycle control genes has been the isolation of *CKS1*, a *S. cerevisiae* homologue of the *S. pombe suc1*$^+$ gene (Hadwiger *et al.*, 1989a). Mirroring the experimental procedure in fission yeast, the *CKS1* gene was isolated by dosage suppression of a temperature sensitive *cdc28* mutation. Antibodies raised against a portion of the *CKS1* gene product are able to coprecipitate an active form of the p34^{CDC28} kinase suggesting that the Cks1 protein is a component of the p34^{CDC28} kinase complexes. Loss of *CKS1* gene function leads to the arrest of mutant cells in G1 with a shmoo morphology similar to that of an arrested *cdc28* mutant (Reed, 1980). Two structural homologues of *suc1*$^+$ have also been identified in human cells (Richardson *et al.*, 1990; Draetta *et al.*, 1987).

Parallel experiments involving dosage suppression of *cdc28* mutations have lead to the isolation of two genes, *CLN1* and *CLN2*, that display limited homology to cyclins (Hadwiger *et al.*, 1989b). A third cyclin homologue has also been isolated from *S. cerevisiae*. The gene was originally identified by the dominant phenotype of the allelic mutations *WHI1-1* and *DAF1-1* which cause the premature execution of START with a consequent reduction in critical cell size and increased resistance to alpha-factor (Sudbery *et al.*, 1980; Nash *et al.*, 1988; Cross, 1988). The *WHI1/DAF1* gene (also known as *FUN10*) has been renamed *CLN3* due to the observation that the three *CLN* gene products perform overlapping functions (Richardson *et al.*, 1989). Cells containing a deletion in any two of the *CLN* genes are viable whilst the triple deletion mutant is unable to complete START. At least one *CLN* gene product is therefore required for p34^{CDC28} kinase activity

at START.

It is thought that the *CLN* gene products are specifically synthesized at START and are rapidly degraded during S-phase. To date however this behaviour has only been reported for the *CLN2* gene product whose level has been monitored in cell lysates from a synchronous culture by immunoblotting analysis (Wittenberg *et al.*, 1990). Nevertheless the *CLN* gene products are regarded as G1 cyclins that are distinct from the G2 cyclins identified in other organisms. A preliminary identification of two distinct G2 cyclins in *S. cerevisiae* has been reported and has contributed to the hypothesis that an S-phase promoting factor (SPM) consisting of the $p34^{cdc2}$ kinase in association with G1 cyclins controls entry into S-phase whilst an M-phase promoting factor (MPF) consisting of the same kinase subunit in association with G2 cyclins controls entry into mitosis (Lewin, 1990). Supporting this is the report that DNA synthesis in *Xenopus* eggs is dependent on the function of a $p34^{cdc2}$-like protein in G1 (Blow & Nurse, 1990).

Each of the *CLN* gene products contains a cluster of PEST sequences at the C-terminus of the proteins (Hadwiger *et al.*, 1989b). PEST regions contain a high content of proline, glutamate, serine and threonine residues and are believed to target proteins for proteolytic degradation (Rogers *et al.*, 1986) and may promote the destruction of the *CLN* gene products after completion of START. The advancement of cells into S-phase by the dominant *WHI1-1*, *DAF1-1* and *CLN2-1* mutations is thought to occur by stabilization of the mutant proteins. Each of the latter gene products contains a C-terminal truncation that removes PEST sequences. The accumulation of the G1 cyclins encoded by the *CLN* genes therefore appears to be rate-

limiting for START. This contrasts with the timing of mitosis in fission yeast where the accumulation of the G2 cyclin encoded by the *cdc13*$^{+}$ is thought not to be rate limiting (Moreno *et al.*, 1989).

The expression of the *CLN* genes has also been examined at the transcriptional level (Wittenberg *et al.*, 1990). Expression of the *CLN1* and *CLN2* genes is transcriptionally regulated with transcript levels rising at the end of G1 and falling again in S-phase. Conversely, the level of *CLN3* transcripts remains constant throughout the cell cycle. A similar difference in transcript levels is also observed during exposure of cells to alpha-factor with *CLN1* and *CLN2* transcript levels falling and *CLN3* transcript levels remaining constant, indicating that *CLN3* expression is regulated at the translational or post-translational level. Insight into the mechanism of cyclin-mediated cell cycle control in response to mating factors has been gained through the isolation of genes whose products are ultimate effectors of the mating pheromone signal transduction pathway (see below). Three such effectors, *FAR1*, *FUS3* and *KSS1* have been isolated that regulate cyclin levels in response to mating pheromone signalling. A mutation in the *FAR1* gene causes an inability of mutant cells to arrest at START in response to mating pheromone despite an intact pheromone signal transduction pathway (Chang & Herskowitz, 1990). In contrast, a *far1*$^{-}$ *cln2*$^{-}$ double mutant regains the capacity for alpha-factor induced arrest leading to the proposal that the *FAR1* gene product specifically inhibits *CLN2* expression in response to mating pheromones and that similar effectors specifically regulate *CLN1* and *CLN3* expression. The *FUS3* gene product is a candidate for a negative regulator of *CLN3* expression (Elion *et al.*, 1990). Mutants in the *FUS3* gene also fail

to arrest at START in response to mating pheromones although the ability to arrest is restored by deletion of the *CLN3* gene. Conversely, overexpression of the *KSS1* gene causes a resistance to alpha-factor induced START arrest that is dependent on *CLN3* expression (Courchesne *et al.*, 1989). Therefore *FUS3* and *KSS1* function antagonistically in the regulation of *CLN3* expression in response to mating pheromone signalling. Both genes display sequence homology to the *ERK1* gene which encodes a protein kinase involved in insulin-stimulated signal transduction in rat cells (Boulton *et al.*, 1990). Comparable effectors involved in the regulation of *CLN1* gene expression have yet to be reported.

1.6 MATING AND THE PHEROMONE SIGNAL TRANSDUCTION PATHWAY

The mating process in yeast is a programme of differentiation that represents a developmental alternative to mitotic reproduction. Cells of *S. cerevisiae* can exist as one of three cell types (reviewed by Herskowitz, 1989). A haploid cell of the a mating type is able to mate with a haploid cell of the opposite alpha mating type to produce a third cell type, the a/alpha diploid. Mating type is governed by the *MAT* locus on chromosome III, with a cells expressing the *MAT*a allele, alpha cells expressing the *MAT*alpha allele and heterozygous diploids expressing both *MAT* alleles. Two silent copies of the mating type alleles, *HMR*a and *HML*alpha, reside at distal ends of chromosome III and are transcriptionally repressed in haploid cells by the products of the *SIR* genes (Rine & Herskowitz, 1987). The *HO* gene encodes a site-specific endonuclease which operates a cassette mechanism for the transposition of mating type information from *HML*a or *HML*alpha to the *MAT* locus (reviewed by Herskowitz & Oshima, 1981).

The expression of the HO endonuclease is transcriptionally regulated by the products of the *SWI* and *SIN* genes which restrict mating type switching to a brief window of activity at START in the cell cycle of mother but not daughter cells (Nasmyth, 1983 and see Herskowitz, 1989). Most laboratory strains used in genetic studies have been deleted for the *HO* gene preventing mating type switching from occurring.

A set of haploid-specific genes are expressed in haploid cells containing either *MAT* allele, including the *RME1* gene which represses the initiation of meiosis (reviewed by Kassir & Simchen, 1989). The *MAT*alpha allele encodes two proteins, the alpha1 protein which positively regulates the transcription of a set of alpha-specific genes (see below) and the alpha2 protein which negatively regulates the transcription of a-specific genes. Haploid cells containing the *MAT*a allele express a-specific genes by default in the absence of the regulatory activities of the alpha1 and alpha2 proteins. In *MAT*a/alpha diploid cells the product of the *MAT*a allele, the a1 protein, forms a complex with the alpha2 protein. The a1-alpha2 regulatory complex represses the transcription of the alpha1 cistron and haploid-specific genes whilst free alpha2 represses the a-specific genes resulting in a diploid cell that has potential for meiotic division.

When cells of the opposite a and alpha mating types are mixed they undergo a process of conjugation (reviewed by Cross *et al.*, 1988). This process involves the reciprocal exchange of mating factors and cell cycle arrest in G1 (Bucking-Throm *et al.*, 1973, Wilkinson & Pringle, 1974), cell adhesion (Betz *et al.*, 1978; Fehrenbacher *et al.*, 1978), formation of a characteristic "shmoo"

morphology (Byers & Goetsch, 1975), plasmogamy (Trueheart *et al.*, 1987; McCaffrey *et al.*, 1987) and karyogamy (Conde & Fink, 1976).

The genes encoding the peptide mating pheromones of *S. cerevisiae* are expressed in a mating type-dependent fashion. Cells of the **a** mating type produce the **a**-pheromone (encoded by the *MF***a**1 and *MF***a**2 genes) which binds to the **a**-factor receptor in the membrane of alpha cells. Cells of the alpha mating type produce the alpha-pheromone (encoded by the *MF*alpha1 and *MF*alpha2 genes) which binds to the alpha-factor receptor in the membrane of **a** cells. Cells of opposite mating type undergo a mutual chemotropic response producing preconjugatory projections towards their prospective partners prior to mating. It is thought that a specific mating partner is selected by its ability to produce the highest local pheromone concentration (a communication and commitment step known as courtship - Jackson & Hartwell, 1990a; 1990b).

The **a**- and alpha-factor receptors are encoded by the *STE3* and *STE2* genes respectively (Hagan, *et al.*, 1986; Nakayama *et al.*, 1985; Burkholder & Hartwell, 1985). Both of the receptors display seven potentially membrane-spanning hydrophobic domains in their predicted protein sequence, a structure similar to that observed in the beta-adrenergic and muscarinic cholinergic receptors and visual rhodopsin which are involved in signal transduction in mammalian cells. The intracellular signal generated by the binding of mating pheromone to its appropriate receptor is thought to be transduced along an intracellular pathway common to both haploid cell types (Bender & Sprague, 1986).

Many of the genes involved in pheromone signal transduction,

including *STE3*, *STE4*, *STE5*, *STE7*, *STE11*, *STE12* and *STE18* were isolated by screening for sterile (*ste*) mutants that are unresponsive to alpha-factor (Hartwell, 1980). Most of these genes (apart from those encoding the pheromone receptors) represent haploid-specific genes which are expressed in both haploid cell types and maintain a low basal level of signal pathway activity even in the absence of mating factors. The *STE4* and *STE18* genes have been shown to display sequence homology to the beta and gamma subunits respectively of the heterotrimeric G proteins that are involved in signal transduction in mammalian cells (Whiteway *et al.*, 1989; and reviewed by Neer & Clapham, 1988). The beta/gamma moiety encoded by the *STE4* and *STE18* is thought to associate with a *S. cerevisiae* G protein alpha subunit homologue which has been isolated on several independent occasions as the product of the *GPA1* (Nakafuku *et al.*, 1987), *SCG1* (Dietzel & Kurjan, 1987), *CDC70* (Jahng *et al.*, 1988) and *DAC1* (Fujimura, 1989) genes. By analogy with mammalian G proteins it has been proposed that binding of pheromone to its receptor may transmit a signal to the associated G protein causing an exchange of GDP for GTP on the alpha subunit which in turn promotes its dissociation from the beta/gamma moiety. As loss of function mutations in the *GPA1* gene result in constitutive pheromone signalling and those in the *STE4* or *STE18* genes result in sterility it is thought that the free beta/gamma moiety functions as the positive tranducer of the pheromone induced signal. In support of this model it has been shown that overexpression of the *GPA1* gene causes a down-regulation of pheromone signalling (Cole *et al.*, 1990) whilst overexpression of the *STE4* gene causes induction of the signalling pathway (Whiteway *et al.*, 1990).

The *STE5* gene functions downstream of the G protein in the signalling pathway although its function is at present unknown. Downstream of *STE5* are the *STE7*, *STE11* and *FUS3* genes, all of which display sequence homology to known protein kinases, although their substrates are as yet unknown (Teague *et al.*, 1986; Elion *et al.*, 1990). At least one target of the pheromone response pathway is a transcription factor encoded by the *STE12* gene (Dolan *et al.*, 1989; Errede & Ammerer, 1989). The Ste12 protein is thought to promote the transcription of pheromone inducible genes that contain a consensus nucleotide motif called the pheromone response element (Kronstad *et al.*, 1987; Van Arsdell *et al.*, 1987). The *FAR1* gene is also an attractive candidate for an ultimate target of the pheromone response pathway (Chang & Herskowitz, 1990). The transcription of the *FAR1* gene is induced in response to mating pheromones and the gene product is thought to promote cell cycle arrest through negative regulation of the expression of the G1 cyclin encoded by the *CLN2* gene. Furthermore expression of the *CLN3* gene is negatively regulated by the *FUS3* gene product in response to pheromone signalling (Elion *et al.*, 1990). The *FAR1* and *FUS3* gene products also play additional roles in plasmogamy and signal transduction respectively.

Several *CDC* genes that are directly involved in the transmission of the mating signal have been isolated by their ability to confer conditional G1 arrest and/or mating competency on mutant cells that lack a functional pheromone receptor. Mutations in the *CDC36*, *CDC39* and *SRM1* genes have been identified that cause a conditional activation of the pheromone response pathway from a stage downstream of the pheromone receptor and at or before the stage of G protein function (Reed,1980; De Barros Lopes *et al.*, 1990; Neiman *et al.*,

1990; Clark & Sprague, 1989). All three genes appear to possess a growth regulatory function in addition to their role in pheromone signalling and it has been suggested that they may encode general regulators of heterotrimeric G proteins in *S. cerevisiae* (De Barros Lopes *et al.*, 1990; Clark & Sprague, 1989). One other gene, *GPA2*, has been isolated in *S. cerevisiae* whose predicted product displays structural homology to the alpha subunits of tripartite G proteins, although its function is as yet unclear (Nakafuku *et al.*, 1988). Mutations in the *CDC72* and *CDC73* genes are also able to confer conditional mating competency on otherwise sterile receptorless mutants and therefore represent further candidates for components of the signal transduction pathway (Reed *et al.*, 1988).

A further aspect of the pheromone response is that in the absence of mating, cells are able to adapt to the presence of mating pheromone and resume mitotic division. Several different mechanisms of densensitization are employed by the cell, involving the function of several different genes. Cells of the a mating type secrete an endopeptidase that is the product of the *BAR1/SST1* gene and which is able to degrade alpha-factor (Ciejek & Thorner, 1979; Chan & Otte, 1982). Mutation of the *SST2* gene renders cells of either mating type hypersensitive to mating pheromone due to a defective adaptation response (Chan & Otte, 1982). Mutations in the *GPA1* gene which activate the signal transduction pathway have also been observed to promote a heightened adaptive response suggesting a desensitizing role for the G protein alpha subunit (Miyajima, *et al.*, 1989; Stone & Reed, 1990) that involves phosphorylation of the beta subunit (Cole and Reed, 1991). The alpha-pheromone receptor itself is thought to play a role in desensitization. Deletion of the C-terminus of the

alpha-receptor leads to supersensitivity to pheromone and the intact receptor has been shown to undergo hyperphosphorylation after exposure to a-factor (Konopka *et al.*, 1988; Reneke *et al.*, 1988). Finally a putative protein kinase encoded by the *KSS1* gene is thought to mediate a further mechanism for adaptation which may involve the activation or stabilization of the G1 cyclin encoded by the *CLN3* gene (Courchesne *et al.*, 1989).

A diploid cell resulting from the successful mating of an a and alpha haploid cell does not synthesize or respond to mating factors and may undergo mitotic or meiotic division depending the nutritional conditions (Malone, 1990).

1.7 THE *RAS*-cAMP NUTRIENT SIGNALLING PATHWAY

An important discovery resulting from genetic studies of the control of cellular proliferation in *S. cerevisiae* has been the role of genes that are homologous to the *ras* oncogene (Powers *et al.*, 1984 and reviewed by Gibbs & Marshall, 1989). Oncogenes of the *ras* family were first identified in acutely transforming RNA tumour viruses. The activation of human *ras* genes has been further linked with certain cancers. Homologues of *ras* have also been identified in the fission yeast *Schizosaccharomyces pombe* and in the fruit-fly *Drosophila melanogaster*. The *ras* oncogenes encode guanine nucleotide-binding proteins that are thought to be involved in cellular signal transduction.

The *ras* homologues *RAS1* and *RAS2* of *S. cerevisiae* are involved in a fermentable-sugar-sensing pathway that regulates the transition from quiescence to active mitotic proliferation through START. The second

messenger mediating nutrient signalling in this pathway is 3',5'-cyclic adenosine monophosphate (cAMP) which is synthesized from ATP by the adenylyl cyclase encoded by the *CYR1/CDC35* gene (Boutelet *et al.*, 1985; Kataoka *et al.*, 1985). cAMP activates a cAMP-dependent protein kinase (cAPK) by binding to its regulatory subunit which is encoded by the *BCY1* gene (Toda *et al.*, 1987a; Cannon *et al.*, 1990) thereby releasing the catalytic subunits which are encoded by the *TPK1*, *TPK2* and *TPK3* genes (Toda *et al.*, 1987b). Phosphorylation of the target substrates of the cAPK is essential for cellular proliferation although the identity of these substrates is largely unknown. Down-regulation of cAPK activity is in turn brought about by the activity of a high and a low affinity phosphodiesterase encoded by the *PDE2* and *PDE1* genes respectively (Sass *et al.*, 1986; Nikawa *et al.*, 1987).

A major role of the *S. cerevisiae RAS* genes is in the stimulation of adenylyl cyclase activity (Kataoka *et al.*, 1984) although there is evidence that they may also serve an additional role in the cell (Toda *et al.*, 1987b; Cameron *et al.*, 1988; Garret & Broach, 1989; Powers *et al.*, 1986; Toda *et al.*, 1988; Michaeli *et al.*, 1989). It has been suggested that the Ras-dependent modulation of adenylyl cyclase activity may be effected via a 70kDa cyclase-associated protein encoded by the *CAP/SRV2* gene (Field *et al.*, 1990; Fedor-Chaiken, 1990). Ras activity, which is restricted to the GTP-bound form of the protein, is itself regulated by upstream components of the signalling pathway. The *CDC25* gene encodes an integral membrane protein which positively regulates Ras activity in response to glucose and which may promote GDP-GTP exchange on the Ras proteins (Garreau *et al.*, 1990; Munder & Kuntzel, 1989; Robinson *et al.*,

1987; Broek *et al.*, 1987; Camonis & Jaquet, 1988, Petitjean *et al.*, 1990). The *IRA1* and *IRA2* gene products are thought to function antagonistically to the *CDC25* gene product promoting down-regulation of Ras activity (Tanaka *et al.*, 1989; Tanaka *et al.*, 1990a). The *IRA1* and *IRA2* genes are homologous to mammalian GAP (ras GTPase activating protein) and may therefore inhibit Ras proteins by increasing their GTPase activity (Ballester *et al.*, 1989, Tanaka *et al.*, 1990b).

Mutational inactivation of the *RAS*-cAMP pathway leads to pleiotropic phenotypes including hyperaccumulation of storage carbohydrates, failure to grow on non-fermentable carbon sources, sporulation in the absence of nutrient deprivation, lithium sensitivity and sensitivity to low glucose concentrations (for example see Cannon *et al.*, 1986; Mitsuzawa *et al.*, 1989; Shilo *et al.*, 1978a). Hyperactivation of the pathway leads to an inability to accumulate storage carbohydrates, failure to sporulate and loss of viability during nitrogen starvation (for example see Kataoka *et al.*, 1984; Cannon *et al.*, 1990; Malone, 1990, but see also Cameron *et al.*, 1988).

1.8 THE *dna26-1* MUTATION

The *dna26-1* mutation was identified during a screen for mutants that display a conditional defect for DNA but not protein synthesis (Dumas *et al.*, 1982). The screen identified a total of ninety seven conditionally lethal, temperature sensitive mutations in 60 complementation groups. Thirty five of the mutations exhibited a "fast stop" phenotype for the arrest of DNA synthesis at the restrictive temperature (36°C).

Mutants displaying a fast stop phenotype were screened for a *cdc*-like unbudded arrest phenotype after a shift to the restrictive temperature (White, 1984; Davies, 1985; Green, 1986). The rationale behind this screen was that the apparent failure to synthesize DNA in these mutants might be the result of a mutational and concerted arrest of cells at START, preventing their entry into S-phase. During reciprocal shift experiments the *dna26-1* mutation was observed to cause a conditional arrest of the cell cycle at a stage interdependent with the stage of arrest of alpha-factor-treated cells (Davies, 1985). A similar result was obtained with a temperature sensitive *cdc28-D1* allele that was also identified in the original screen by Dumas and his colleagues and used in the above experiments for the purposes of comparison. Arrest of the cell cycle at the pheromone sensitive step is a criterion for a START mutant and it was therefore tentatively concluded that the *dna26-1* mutation causes an arrest of the cell cycle at START.

In order to verify that *dna26-1* is a novel START mutation, a series of complementation tests were carried out in which the *dna26-1* mutant strain JL448 was crossed with a collection of mutants that display either a START arrest, START arrest-like, or general *cdc* phenotype. The *dna26-1* mutation was shown to complement *cdc1-31* (White, 1984) and all published mutants displaying a START-like phenotype (Green, 1986) on YEPD agar at 36°C (Table 1.2).

Physiological experiments on the primary mutant strain JL448 indicated that the *dna26-1* mutation was causing a Class I-type START arrest. (Reed, 1980). Mating experiments revealed that JL448 retained a high conjugation efficiency at 34°C (Davies, 1985). JL448 was also shown to maintain a high rate of uptake of radiolabelled

Allele	Result with *dna26-1*	Allele	Result with *dna26-1*
cdc28-D1	+	*cdc37-1*	+
cdc36-16	+	*cdc39-1*	+
cdc60-1	+	*cdc61-1*	+
cdc62-1	+	*cdc63-1*	+
cdc64-1	+	*cdc65-1*	+
cdc66-1	+	*cdc67-1*	+
cdc68-1	+	*prt1-1**	+
*ils1-1**	+	*mes1-1**	+
*alg1-1**	+		

TABLE 1.2 Complementation data from crosses of the *dna26-1* mutant with mutant strains that display a START arrest phenotype

Complementation tests were carried out on YEPD agar at 36°C. (+) - positive complementation.

* - data from Green, 1986. Other data from Davies, 1985.

amino acids at 36°C suggesting that protein synthesis continued in this strain at the restrictive temperature (although these experiments did not specifically measure incorporation of amino acids into cellular protein - Green, 1986). It was also observed that the *dna26-1* mutant did not form shmoos at the restrictive temperature suggesting that the *DNA26* gene is not directly involved in the mating pheromone signal transduction pathway (Davies, 1985).

1.9 AIMS OF THE PROJECT

The aims of the project were to isolate the wild type *DNA26* gene by molecular cloning and to utilize the cloned gene to investigate its functional role in the control of the *S. cerevisiae* cell cycle. A further intention was to map the *DNA26* locus to a chromosome in an attempt to eliminate possible allelism with previously identified genes and mutations. A parallel aim was to further characterize the effect of the *dna26-1* mutation on cellular physiology and START.

CHAPTER 2 : GENETIC ANALYSIS OF THE *dna26-1* MUTATION

2.1 INTRODUCTION

CLASSICAL GENETIC ANALYSIS

The primary *dna26-1* mutant strain JL448 was identified after mutagenic treatment of the wild type haploid strain A364A in a screen for mutants defective in DNA synthesis (Dumas *et al.*, 1982). Such mutagenic treatment (in this case employing ethyl methane sulphonate) is capable of generating more than one mutation in a single genome. The phenotype of JL448 at 25°C on rich medium was apparently similar to a wild type strain. However it was considered possible that the temperature sensitive phenotype ascribed to the *dna26-1* mutation might result from the interactive effects of more than one mutation.

Standard procedures have been described for determining whether a particular mutant phenotype is caused by the effect of a single mutation (Mortimer & Hawthorne, 1969). These classical genetic techniques are based upon the prediction by Mendel's first law that the meiotic products of a diploid containing a difference in a single pair of alleles will display a 2:2 segregation ratio of the two allelic loci. It is possible to construct a diploid yeast strain which is heterozygous for the *dna26-1* mutation by crossing the mutant strain JL448 to a haploid wild type strain of opposite mating type. Such a diploid would be amenable to classical tetrad analysis providing a means of determining whether the temperature sensitive G1-arrest phenotype of JL448 is attributable to a single mutation.

Tetrad analysis allows the pattern of segregation of various genetic markers that are present in a diploid mutant to be analysed. Backcrossing a strain that has been subjected to mutagenic treatment into a wild type genetic background also has other benefits.

Unwanted mutations that may influence the effect of the allele of interest may be diluted out by repeated selection for a particular phenotype. The required mutation can be simultaneously introduced into a well characterized genetic background. Furthermore, the wild type parent used for backcrossing can serve as a valid control for comparison with the congenic strain displaying the mutant phenotype. Backcrossing also allows the dominant or recessive nature of a mutation to be evaluated in the presence of its wild type allele.

In contrast to the elimination of unwanted mutations in a strain, backcrossing procedures can also permit the introduction of useful genetic markers into a genome. Vectors employed in yeast molecular biology have generally been constructed to carry genes involved in a specific biosynthetic pathway. Introduction of the allelic auxotrophic mutation into a transformation host strain is therefore a prerequisite for vector selection.

Cell type can have an effect on the phenotype conferred by some mutations. Tetrad analysis can generate strains for the analysis of a mutation in a *MAT***a** / *MAT*alpha diploid or in a *MAT***a** or *MAT*alpha haploid cell. The temperature sensitive phenotype caused by the *dna26-1* mutation can be distinguished from auxotrophic mutations present in the same strain and the phenotype conferred by the mating type specific genes. Tetrad analysis therefore provides a means of comparing the pattern of segregation of the temperature sensitive trait in the *dna26-1* mutant with that of known auxotrophic and mating type markers. The linkage relationship of the various mutations can therefore also be determined (Mortimer & Hawthorne, 1969). Mendel's second law predicts independent assortment of markers residing on

different chromosomes. Any deviation from this law in the pattern of segregation of the *dna26-1* mutation compared with another specific marker would be expected to yield evidence of its genomic location.

It was therefore decided to backcross the *dna26-1* mutant JL448 into a wild type haploid strain. The aim of this procedure was to establish that the temperature sensitve phenotype displayed by JL448 was due to a single mutation, *dna26-1*, and to purify this mutation from other possibly contaminating mutations. The backcrossing procedure was also designed to construct a *dna26-1* mutant suitable for cloning work.

Genetic interactions of the *dna26-1* mutation with another recently characterized mutation causing conditional G1 arrest was also looked for. The *srm1-1* mutation was isolated as a conditional supressor of mutations in the *STE3* gene (Clark & Sprague, 1989). *STE3* encodes the a pheromone receptor expressed in cells of mating type alpha. The *srm1-1* mutation restores mating ability to *ste3* mutants at the restrictive temperature and also causes temperature sensitive G1 accumulation of *srm1-1* mutant cells. This phenotype suggested that the *srm1-1* and *dna26-1* mutations might be allelic, warranting complementation analysis.

MOLECULAR GENETIC ANALYSIS

Many yeast genes have been cloned by functional complementation of a mutation by a plasmid-borne copy of the wild type gene (Struhl, 1983). Targetted selection of clones at high gene dosage has been achieved through the use of high copy vectors containing the origin of replication for the endogenous *S. cerevisiae* 2μm plasmid (Rine *et*

al., 1983). By this means, biosynthetic genes have been cloned by the ability of transformants to overcome growth inhibitors when the cloned gene was expressed at high dosage. Dosage suppression of mutations by non-allelic genes carried on high copy plasmids has also been employed as an alternative cloning strategy (for example see Hadwiger *et al.*, 1989a and 1989b; Hayles *et al.*, 1986a).

Several genes involved in the control of cellular proliferation in *S. cerevisiae* are thought to function in the cAMP-mediated signal transduction pathway. The genetic interactions taking place in this pathway have been investigated by suppressor analyses. These have included screening for extragenic suppressor mutations (for example see Cannon *et al.*, 1986) or for dosage suppressors of certain mutations in the pathway. In studies of the latter type, downstream elements with a positive function in signal transduction have been shown to display dosage suppression of mutations in upstream positive elements in the pathway. For example the *TPK1* gene of *S. cerevisiae* encodes one of the three catalytic subunits of the cAMP-dependent protein kinase (cAPK). It has been demonstrated that overexpression of this gene on a high copy plasmid can cause the suppression of mutations in both the *CDC25* and *CDC35(CYR1)* genes (Toda *et al.*, 1987b). Furthermore, overexpression of the *CYR1* gene encoding the structural gene for adenylyl cyclase in *S. cerevisiae* has been shown to cause dosage suppression of temperature sensitive mutations in the *CDC25* and *RAS2* genes (Toda *et al.*, 1988; Mitsuzawa *et al.*, 1989).

Downstream elements of the cAMP-mediated signal transduction pathway at high gene dosage can antagonise hyperactivating mutations in upstream positive elements. For example the dominant *RAS2*^{val19} mutation, which itself can cause dosage suppression of a mutation in

the *CDC25* gene, has been shown to be dosage suppressed by overexpression of the *PDE1* or *PDE2* gene (Sass *et al.*, 1986; Nikawa *et al.*, 1987). *PDE1* and *PDE2* encode respectively the low and high affinity phosphodiesterases of *S. cerevisiae*. *PDE2* has also been shown to cause dosage suppression of a mutation in the *IRA1* gene (Tanaka *et al.*, 1989). The function of the *IRA1* gene is in the down-regulation of *RAS*-mediated activation of adenylyl cyclase.

Two further genes, *SCH9* (Toda *et al.*, 1988) and *SCD25* (Boy-Marcotte *et al.*, 1989) have also been isolated as dosage suppressors of mutations in the RAS-cAMP pathway. The *SCH9* gene encodes a protein kinase that is structurally and functionally related to the catalytic subunits of the cAPK in *S. cerevisiae* and may also have a role in the control of proliferation. The *SCD25* gene displays structural homology to the C-terminal portion of the *CDC25* gene. The C-terminal portion of *SCD25* expressed on a high copy plasmid can suppress mutations in the *CDC25* gene by promoting GDP-GTP exchange on Ras proteins (Crechet *et al.*, 1990) thereby activating adenylyl cyclase.

Temperature sensitive mutations in the positive elements of the cAMP-mediated signal transduction pathway, including *cdc25*, *cdc35*, *ras1* *ras2*^{ts}, and *tpk1*^{ts}, cause G1 arrest at START. This conditional phenotype is similar to that caused by the *dna26-1* mutation. With two objectives in mind the *dna26-1* mutant TDE/16A was separately transformed with high copy plasmids bearing genes involved in the RAS-cAMP pathway. Firstly, these experiments allowed elimination of the possibility that the cloned genes employed might be allelic to *dna26-1*. Secondly, the experiments tested for possible interaction of the *dna26-1* mutation with genes involved in Ras/cAMP-mediated signal transduction.

During the course of the studies on the *dna26-1* mutation, another mutation of *S. cerevisiae* was reported that could also cause a G1 arrest phenotype. The *CKS1* gene was identified as a dosage suppressor of mutations in the *CDC28* gene (Hadwiger *et al.*, 1989a). *CKS1* is homologous to the *Schizosaccharomyces pombe suc1*$^+$ gene and is thought to comprise a subunit of the Cdc28 kinase complex. Haploid spores containing a disruption in the *CKS1* gene germinate, but produce only about 50 cells. These cells arrest in the G1 phase of the cell cycle. Similarly, cells with a genomic disruption in the *CKS1* gene but also containing a copy of *CKS1* on an autonomously replicating plasmid arrest in G1 upon plasmid loss.

To eliminate *CKS1* as a possible allele of *DNA26* a plasmid carrying the *CKS1* gene was obtained. The genetic relationship between the *CKS1*$^+$ gene and *dna26-1* mutation was assessable by transformation of TDE/16A with the plasmid-borne *CKS1* gene.

2.2 MATERIALS AND METHODS

STRAINS

The strains of *S. cerevisiae* used in this section of the work are listed in Table 2.1.

MEDIA

All media were obtained from Lab-M except where stated. All chemicals were obtained from Sigma or BDH. 1.6%(w/v) Lab-M tissue culture agar was included in solid media.

SD

4%(w/v) dextrose, 0.67%(w/v) yeast nitrogen base without amino acids (Difco). Nutrient supplements were added to minimal, selection and dropout media to the following final concentrations :-

Adenine	20µg/ml	(from a 1.2mg/ml stock)
Uracil	20µg/ml	(from a 2.4mg/ml stock)
L-Tryptophan	20µg/ml	(from a 2.4mg/ml stock stored at 4°C)
L-Histidine	20µg/ml	(from a 2.4mg/ml stock stored at 4°C)
L-Arginine	20µg/ml	(from a 2.4mg/ml stock stored at 4°C)
L-Methionine	20µg/ml	(from a 2.4mg/ml stock stored at 4°C)
L-Tyrosine	30µg/ml	(from a 0.9mg/ml stock)
L-Phenylalanine	50µg/ml	(from a 3.0mg/ml stock)
L-Leucine	30µg/ml	(from a 3.6mg/ml stock)
L-Lysine	30µg/ml	(from a 3.6mg/ml stock stored at 4°C)

Supplement stock solutions were stored at room temperature except where indicated.

Strain	Mating Type	Genotype	Source
JL448	a	*dna26-1 ade1 ade2 ura1 his7 lys2 tyr1 gal1*	L.D.
MD40/4C	alpha	*ura2 leu2-3/112 his3-11/15 trp1*	M.T.
A33	a	*ura3-251/373 leu2-3/112 his3-11/15 trp1 lys⁻*	M.T.
TDE/16A	a	*dna26-1 ade2 trp1 leu2-3/112 ura3-251/373*	D.E.
RC631	a	*sst2-1 rme ade2 his6 met1 ura1 can1 cyh2*	R.C.
MIS1	alpha	*sst2-1 rme his6 met1 can1 cyh2*	N.S.
SY1115	alpha	*srm1-1 ura3 trp1 leu2-3/112 his7*	G.S.
DBY747	a	*his3-1 leu2-3/112 ura3-52 trp1*	D.B.
DBY746	alpha	*his3-1 leu2-3/112 ura3-52 trp1*	D.B.
X4003/5B	a	*ade1 leu2 met2 ura3 his4 trp5*	P.S.
TD4	alpha	*dna26-1 ade1 ade2 his3-11 lys2 leu2-3/112 ura1*	D.E.
SC8	alpha	*ade2-6*	A.W.
SC2	alpha	*ade1.0*	A.W.
SRA/364/47	alpha	*ade1 tyr1 lys2 ade2*	D.E

TABLE 2.1 Yeast Strains Used in the Genetic Analysis of *dna26-1*

The genotypes of the yeast strains used during the genetic analysis of the *dna26-1* mutation are shown with the source from which each was obtained. L.D., Lawrence Dumas; M.T., Mick Tuite; R.C., Russell Chan; N.S., Nigel Stenner; G.S., George Sprague; P.S., Peter Sudbery; D.B., David Botstein; A.W., Alan Wheals; D.E., constructed by author.

Dropout media consisted of SD medium containing all, none, or all but one of a range of necessary supplements encompassing the auxotrophic requirements of the parents from which the strain to be tested was derived.

YEPD

2%(w/v) dextrose, 2%(w/v) mycological peptone, 1%(w/v) yeast extract.

SPORULATION MEDIA

Presporulation and sporulation plates contained media described by Sherman *et al.*, (1986). Liquid presporulation (YPA) and sporulation (SPM) were prepared as described by Shuster & Byers (1989).

Presporulation Medium (used in plates)

10%(w/v) dextrose, 0.8%(w/v) yeast extract, 0.3%(w/v) mycological peptone.

Sporulation Medium (used in plates)

1%(w/v) potassium acetate. Where required supplements were added to 25% of the concentration normally added to minimal medium.

YPA

1%(w/v) potassium acetate, 1%(w/v) yeast extract, 2%(w/v) mycological peptone.

SPM

0.3%(w/v) potassium acetate, 0.02%(w/v) raffinose.

YEAST GENETIC METHODS

Mating Type Screening

The mating type of yeast strains was determined using a halo assay developed from the assay described by Fink & Styles (1972). One large colony of an *sst2-1* mutant strain (Chan & Otte, 1982) was suspended in 5ml of YEPD and briefly vortexed. 125µl of the suspension was inoculated into 5ml of molten YEPD (0.7% agar) that had been cooled to 45°C. The molten agar was immediately overlaid onto a YEPD plate and allowed to solidify. The strains to be screened for mating type were patched onto the surface of the overlay and incubated at 25°C. After 24-48hr the presence or absence of a clear zone of growth inhibition around each patch was noted. Tests were carried out using supersensitive *sst2-1* strains of both mating type.

Mating Conditions

Haploid strains of opposite mating type were patched and mixed on a YEPD agar plate and incubated at 25°C for 5-8hr.

Diploid Selection

Parent haploid strains carrying complementary auxotrophic markers were used in mating experiments. Diploids were therefore selected on the basis of complementation of auxotrophic markers. Selection was carried out on SD plates incubated at 30°C for 2-3 days. Appropriate supplements were added when necessitated by homozygosity for particular auxotrophic mutations.

Sporulation Procedure

Sporulation was routinely carried out on solid medium. A single diploid colony was streaked onto presporulation agar and incubated at

30°C for 48hr. A heavy inoculum was then transferred to sporulation agar by streaking. Samples were checked for ascus formation by light microscopy after 3-5 days at 30°C.

Where poor frequencies of sporulation were obtained on solid medium, an alternative liquid medium was employed. A single diploid colony was inoculated into 25ml of YPA in a 100ml conical flask. The culture was incubated in a shaking water bath at 30°C until a cell density of $2X10^7$/ml had been reached (36hr). The cells were harvested by centrifugation at 3150g for 10min and washed twice in sterile H_2O. Finally, the cells were resuspended in 25ml of SPM and incubated at 30°C with vigorous shaking. Ascus formation was monitored by light microscopy after 2-5 days.

ISOLATION OF SPORE CLONES

Tetrad Dissection

Sporulating cells were suspended in 30µl of β-glucuronidase (Sigma, G-0762). Cells were scraped from solid medium using a sterile toothpick or harvested from liquid medium using a microfuge. The suspension was incubated at 37°C for 10-35min to allow for digestion of the ascus wall, then diluted with 50µl of YEPD. A 10µl aliquot of the suspension was dripped onto a supplemented YEPD plate. Tetrads were dissected using a Singer micromanipulator and a fine glass loop constructed using a de Fonbrune microforge apparatus. Isolated spores were germinated at 25°C for 2-4 days and streaked for single colonies on YEPD plates.

Random Spore Analysis (After Dawes & Hardie, 1974).

Sporulating cells were scraped from a sporulation plate, suspended

in 50µl of β-glucuronidase and incubated at 37°C for 30min. The suspension was sonicated for 10secs in an MSE sonicator and placed on ice for 3min. An equal volume of ice-cold diethyl ether was added and the tube shaken vigorously in iced-water for 165secs. 50µl of the suspension was added to 350µl of YEPD in a fresh microfuge tube. 100µl aliquots of the suspension were spread onto YEPD plates and incubated at 25°C for 3 days. Germinated haploid, adenine auxotrophic colonies, identified by their small colony size and red pigmentation were streaked individually onto YEPD agar.

SCREENING FOR AUXOTROPHIC REQUIREMENTS

Separate strains were streaked for single colonies on dropout plates and incubated at 25°C for 3 days.

CLASSICAL COMPLEMENTATION ANALYSIS

Complementation of Auxotrophic Mutations

Haploid strains of opposite mating type were mated on SD medium supplemented with 1%(w/v) YEPD. The supplement whose biosynthetic gene was under analysis was omitted from the medium whilst all other supplements required for diploid growth were added at the normal concentration. The haploid strains were streaked at right angles onto the medium. Positive or negative complementation was scored by assessing growth at the intersection of the two streaks after 4 days at 25°C.

Complementation of Temperature Sensitive (ts^-) Mutations

Diploids constructed by mating ts^- parents were selected by complementation of auxotrophic markers at 25°C and mating type

testing. The diploid was then streaked for single colonies on YEPD agar at 25°C and 36°C. Comparison of proliferation at the permissive and non-permissive temperature was made after 4 days of incubation.

SCREENING FOR THE *cdc* PHENOTYPE OF THE *dna26-1* MUTANT

Mass Screening

Individual strains were streaked for single colonies on two separate YEPD plates. The plates were incubated for 4 days; one at 25°C and one at 36°C. Temperature sensitive and control parental wild type strains were simultaneously grown for comparison with test strains.

Temperature Shift Experiments

These were carried out to establish the terminal phenotype and speed of cell cycle arrest of certain ts^- strains at the restrictive temperature. For each strain, a single colony was inoculated into 10ml of YEPD and incubated at 25°C in a shaking water bath until the culture had reached stationary phase ($2X10^8$ cells/ml). The speed of the shaking water bath was maintained at 106rpm throughout the experiment. The starter culture was used to inoculate 50ml of YEPD and the culture was incubated overnight at 25°C with shaking. When the culture had reached a density of $1-5X10^6$ cells/ml the culture was shifted to 36°C in a second shaking water bath. The culture budding index was determined immediately after the temperature shift and subsequently at hourly intervals for a period of 4-5 hours. Cell samples were fixed by adding formaldehyde to the medium to a final concentration of 4%(v/v). The cells were briefly sonicated prior to the determination of budding index. At least 200 cells were visually

inspected and the budding index calculated using the following equation :

B.I. = (Number of budded cells) / (Total number of cells)

TEST FOR SUPPRESSION OF TDE/16A BY CLONED GENES

a) *CKS1*

Plasmid pJH4-67-1 (a gift from Steven Reed) was extracted from *E.coli* by the midi-prep. method (see section 4.2). The *dna26-1* mutant TDE/16A was transformed to uracil independence with pJH4-67-1 by the alkali cation method (see section 4.2). Putative transformants were initially incubated on selective SD medium at 25°C. After 24hr at 25°C half of the transformation plates were transferred to 36°C and incubation of both sets of plates was continued for a further 7 days before comparison of colony formation.

b) Genes of the *RAS*-cAMP Signalling Pathway

Plasmid DNA was prepared by the midi-prep. method and transformation of intact yeast cells was carried out by the alkali cation method (see section 4.2). The plasmids carrying genes that function in the *RAS*-cAMP signalling pathway and that were used to transform TDE/16A are described in Table 2.2. The presence or absence of transformant colonies was determined on selective SD medium after incubation at either 25°C or 36°C for 10days.

PLASMID	CONSTRUCTION	SOURCE	REFERENCE
YEp351-*CDC25*	5.5Kb *Sal*I-*Pvu*II fragment in YEp351	K.T.	Petitjean *et al.*, 1990
pAP5410 (*CDC25*)	5.5kb *Sal*I-*Pvu*II fragment in YEp24	K.T.	Petitjean *et al.*, 1990
YEp(*RAS2*)-1	6.7kb *Sau*3A fragment in YEp13	M.W.	Kataoka *et al.*, 1984
YEp(*RAS2*^{val19})	2.3kb *Hind*III-*Eco*RV fragment in *Hind*III-*Pvu*I fragment of YEp13	M.W.	Kataoka *et al.*, 1984
pMF216 (*SRV2*)	4.0kb *Hind*III fragment in YCp50	J.B.	Fedor-Chaiken *et al.*, 1990
pCYR1-2 (*CYR1*)	10.1Kb *Sau*3A fragment in YCp50	M.W.	Kataoka *et al.*, 1985
pBCY1-3 (*BCY1*)	4.2kb *Bam*HI fragment in YEp24	M.W.	Toda *et al.*, 1987a
YEpTPK1 (*TPK1*)	2.4kb *Hind*III-*Sph*I fragment in YEp13	M.W.	Toda *et al.*, 1987b

TABLE 2.2 Plasmids Used to Transform TDE/16A

The above plasmids were separately used to transform the *dna26-1* mutant TDE/16A. Each plasmid carries a gene that is thought to be involved in the Ras-cAMP-mediated signal transduction pathway as well as a biosythetic marker required for plasmid selection. Source of plasmids: K.T., Kelly Tatchell; M.W., Michael Wigler; J.B., James Broach.

2.3 RESULTS

BACKCROSSING OF THE *dna26-1* MUTANT INTO A WILD TYPE STRAIN

The strain MD40/4C was chosen as the wild type parent into which the *dna26-1* mutant could be backcrossed in order to check for Mendelian segregation of the temperature sensitive mutation. MD40/4C displays a wild type proliferative response at 36°C. In addition, MD40/4C is also amenable to high efficiency transformation by heterologous DNA in the form of bacterial/yeast shuttle vectors (A. Spalding, personal communication).

Diploids derived from the cross between MD40/4C and the primary *dna26-1* mutant JL448 were induced to sporulate and 12 tetrads were dissected by micromanipulation. 10 asci yielded 4 viable spores and 2 asci yielded 3 viable spores. Spore clones were screened for auxotrophic requirements (data not shown), mating type and temperature-dependent G1 arrest (Table 2.3).

Progeny from this first backcross were chosen that were both leucine requiring and temperature sensitive for proliferation. These selected progeny strains were subjected to temperature shift analysis. Exponentially growing cells were shifted from 25°C to 36°C. The budding index for each culture was monitored for several hours and compared with that of the parental strains (Table 2.4).

Cells of strain TD/1100C (*MAT*alpha *dna26-1 lys2 trp1 leu2-3/112 tyr1* His- Ade^-) displayed a rapid decrease in budding index after the shift to 36°C. The terminal phenotype of TD/1100C was comparable to that of the temperature sensitive parent JL448. TD/1100C was therefore selected as the temperature sensitive parent for further

MARKERS	MARKER SEGREGATION RATIOS		
	2:2	3:1	1:3
4 Germinated Spores			
ts^+ : ts^- *	5	2	3
*MAT*a : *MAT*alpha	9**	0	0
	2:1	1:2	
3 Germinated spores			
ts^+ : ts^- *	2	0	
*MAT*a : *MAT*alpha	1	1	

TABLE 2.3 Segregation Data from the Cross Between MD40/4C and JL448

The number of asci displaying a particular pattern of segregation is shown for the segregation of temperature sensitive and mating type markers. Segregation patterns were recorded for tetrads from which 3 or 4 spores germinated. Spore clones were streaked onto YEPD plates and incubated at 36°C for 4 days for the determination of temperature sensitivity. Mating type was determined by the halo assay.

* ts^+ - wild type proliferative response at 36°C.
ts^- - temperature sensitive arrest of proliferation at 36°C.

** Mating type not determined for the spore clones from one ascus.

STRAIN	TIME AFTER TEMPERATURE SHIFT (Min.)			
	0*	60	120	240
TD/400A	0.95	0.39	0.38	0.25
TD/800A	1.00	0.50	0.31	0.15
TD/900A	1.00	0.50	0.35	0.25
TD/1100C	1.00	0.30	0.33	0.14
TD/1300B	0.90	0.70	0.53	0.66
MD40/4C	1.00	0.70	0.62	0.60
JL448	1.00	0.30	0.50	0.20

TABLE 2.4 Culture Budding Indices of Temperature Sensitive Segregants

A selection of temperature sensitive (ts^-) segregants from the cross between MD40/4C and JL448 were subjected to temperature shift analysis. Exponential cultures were shifted from 25°C to 36°C at 0min. Culture budding index was measured at hourly intervals by microscopic examination. Strains MD40/4C and JL448 were included as wild type and ts^- controls respectively.

* Samples taken at the time of the temperature shift (t=0) from each culture were not sonicated due to practical difficulties. Overestimation of the budding index of these samples may therefore have resulted. Samples taken at all other time points were sonicated.

backcrossing.

Introduction of the *ura3* mutation into the *dna26-1* Genetic Background

During preparation for the intended cloning work, a gene library was obtained based on the *URA3* plasmid YCp50 (Appendix 1(A)). It was therefore decided to introduce a *ura3* mutation into a *dna26-1* genetic background. The temperature sensitive F1 strain TD/1100C from the first backcross was mated with the uracil auxotrophic strain A33. A33 displays a wild type proliferative response to temperature and contains the double mutation *ura3-251/373* conferring uracil dependency.

Diploids resulting from the cross between TD/1100C and A33 were induced to sporulate and 9 asci were dissected by micromanipulation. 3 tetrads yielded 4 viable spores and 6 tetrads yielded 3 viable spores revealing a high frequency of spore inviability. The segregation pattern of auxotrophic markers (data not shown) and markers for mating type and temperature sensitivity (Table 2.5) was examined. Three strains that were both temperature sensitive and uracil requiring were selected for temperature shift analyses.

Both strain DE/7B and DE/10B displayed a marked fall in the culture budding index when shifted to the restrictive temperature (Table 2.6). The budding index for each culture after 4 hours at 36°C was 0.12 and 0.16 for DE/7B and DE/10B respectively. DE/7B displayed the largest fall in budding index at 36°C. This response was also rapid, the budding index reaching a value of 0.08 after 3 hours. However, a very low level of sporulation was achieved by diploids derived by crossing DE/7B with the wild type parent strain MD40/4C (data not shown). In contrast, diploids derived from a cross between the

MARKERS	MARKER SEGREGATION RATIOS		
	2:2	1:3	
4 Germinated spores			
ts$^+$: ts$^-$ *	2	1	
*MAT*a : *MAT*alpha	2**	-	
	1:2	2:1	3:0
3 Germinated spores			
ts$^+$: ts$^-$ *	2	1	3
*MAT*a : *MAT*alpha	4	2	0

TABLE 2.5 Segregation Data from the Cross Between TD/1100C and A33

The segregation pattern of markers for temperature sensitivity and mating type are shown for tetrads from which 3 or 4 spores germinated. Spore clones were streaked onto YEPD plates and incubated at 36°C for 4 days for the determination of temperature sensitivity.

* ts$^+$ - wild type proliferative response at 36°C.
ts$^-$ - temperature sensitive arrest of proliferation at 36°C.

** Mating type not determined for the spore clones from one ascus.

STRAIN	TIME AFTER TEMPERATURE SHIFT (Min.)				
	0	60	120	180	240
DE/7B	0.66	0.30	0.16	0.08	0.12
DE/10B	0.48	0.47	0.35	0.23	0.16
DE/8C	0.50	0.24	0.39	0.33	0.32
A33	0.39	0.37	0.52	0.56	0.52
JL448	0.71	0.34	0.26	0.12	0.05

TABLE 2.6 Culture Budding Indices of Temperature Sensitive Segregants

A selection of temperature sensitive segregants from the cross between A33 and TD/1100C were subjected to temperature shift analysis. Exponential cultures were shifted from 25°C to 36°C at 0min. Culture budding index was measured at hourly intervals by microscopic examination. Strains A33 and JL448 were included as wild type and temperature sensitive controls respectively.

strain DE/10B (*MAT*a *dna26-1 trp1 ura3-251/373 leu2-3/112* Lys- Ade- His-) and MD40/4C displayed a high sporulation frequency. DE/10B was therefore preferred as the temperature sensitive parent for further backcrossing.

Further Backcrossing into MD40/4C

The mutant strain DE/10B was crossed with the wild type strain MD40/4C and the resultant diploid induced to sporulate. Haploid progeny were isolated by both tetrad dissection and random spore analysis.

a) Tetrad Dissection

A total of 26 tetrads were dissected with 22 asci yielding 4 viable spores and 4 asci yielding 3 viable spores. Spore clones were screened for temperature sensitivity, mating type and auxotrophic requirements (Table 2.7). Segregation of the temperature sensitive (ts^-) and temperature resistant (ts^+) phenotypes showed a ratio of $2ts^+:2ts^-$ segregants in 18 out of 22 complete tetrads. This suggested the presence of a single temperature sensitive mutation or two that were closely linked (see section 2.4, Discussion).

Segregation of mating type showed a ratio of 2*MAT*a:2*MAT*alpha segregants in all 22 complete tetrads as expected for Mendelian segregation of the alleles resident at the two parental *MAT* loci. A total of 18 *MAT*a ts^-, 18 *MAT*a ts^+, 18 *MAT*alpha ts^- and 18 *MAT*alpha ts^+ segregants were scored in the 18 asci showing a 2:2 segregation of temperature sensitive to temperature resistant spores (not shown). Furthermore, among these 18 asci studied for the segregation of mating type and temperature sensitivity, the ratio of ascal classes was 3PD:3NPD:12TT, indicating that the temperature sensitive mutation

TABLE 2.7 Segregation Data from the Cross Between MD40/4C and DE/10B

The segregation data are shown for tetrads from which 3 or 4 spores germinated. The segregation of markers for temperature sensitivity, mating type and auxotrophic requirements were analysed. Spore clones were streaked onto YEPD plates and incubated at 36°C for the determination of temperature sensitivity. Mating type was determined by the halo assay and auxotrophic requirements were determined by replica-plating individual strains onto dropout medium.

* ts^+, wild type proliferation response at 36°C

ts^-, temperature sensitive arrest of proliferation at 36°C

\# Auxotrophic mutations of uncertain identity segregating

** Identity of mutations deduced from parental genotypes

\#\# *ura3* and leaky *ura2* mutations segregating

MARKERS	MARKER SEGREGATION RATIOS			
	2:2	1:3	3:1	0:4
4 Germinated Spores				
ts$^+$: ts$^-$ *	18	3	1	0
*MAT*a : *MAT*alpha	22	0	0	0
Ade$^+$: Ade$^-$ *	21	0	1	0
TRP1 : *trp1* **	0	0	0	22
LEU2 : *leu2* **	0	0	0	22
His$^+$: His$^-$ *	5	14	0	3
Ura$^+$: Ura$^-$ **	18	3	0	1
Lys$^+$: Lys$^-$ *	20	0	2	0
	1:2	2:1	3:0	0:3
3 Germinated Spores				
ts$^+$: ts$^-$ *	1	2	0	1
*MAT*a : *MAT*alpha	3	1	0	0
Ade$^+$: Ade$^-$ *	2	2	0	0
TRP1 : *trp1* **	0	0	0	4
LEU2 : *leu2* **	0	0	0	4
His$^+$: His$^-$ *	1	2	0	1
Ura$^+$: Ura$^-$ **	4	0	0	0
Lys$^+$: Lys$^-$ *	2	2	0	0

was not linked to the *MAT* locus.

Only 1 tetrad out of 22 examined failed to display 2:2 segregation of the unidentified adenine auxotrophic mutation. This suggested the segregation of a single mutation conferring adenine dependence. A rare gene conversion event may have caused the 3Ade$^+$:1Ade$^-$ segregation pattern observed in one tetrad. All spores from each ascus examined were auxotrophic for both leucine and tryptophan. This was expected as the *leu2* and *trp1* mutations were present in both haploid parental strains in the cross between DE/10B and MD40/4C. The diploid from which the spores were derived was therefore homozygous for both the *leu2* and *trp1* mutation.

The segregation of histidine dependence was complex. The *his7* mutation originally derived from strain JL448 and the *his3* mutation derived from MD40/4C were probably both randomly segregating in this cross. The observation of a large number of tetrads displaying a 1His$^+$:3His$^-$ ratio of segregation was consistent with the previous assignment of the *HIS7* and *HIS3* genes to different chromosomes (Mortimer & Schild, 1980). An unexpected pattern of segregation was observed for the *ura2* and *ura3* mutations present in this cross. Although the *URA2* and *URA3* genes are unlinked, 18 out of the 22 tetrads examined displayed a ratio of 2Ura$^+$:2Ura$^-$ segregants. This can be explained by the leakiness of the *ura2* mutation. During the backcrossing process it was observed that the parental strain MD40/4C carrying the *ura2* mutation was capable of limited growth on medium lacking uracil. It is possible that the leakiness of the *ura2* mutation was further increased in the genetic background produced by the cross with DE/10B. Thus segregants containing the functional *URA3* allele were therefore probably capable of uracil independent

growth regardless of the presence of the *ura2* mutation. By this interpretation the segregation pattern observed for Ura$^-$ phenotype was essentially a result of the segregation of the *ura3* allele derived from DE/10B.

Finally, 20 out of 22 tetrads displayed a segregation pattern for lysine dependence of 2Lys$^+$:2Lys$^-$ spores whilst 2 tetrads showed a ratio of 3Lys$^+$:1Lys$^-$ segregants. These data suggested the segregation of a single *lys*$^-$ mutation. Two independent gene conversion events may have caused the 3:1 segregation ratios observed in two tetrads.

Random Spore Analysis

A total of 148 haploid progeny from the cross between MD40/4C and DE10B were isolated after germination on supplemented YEPD plates during 3 days of incubation at 25°C. Initial selection was on the basis of red colony colour indicating the presence of a recessive adenine auxotrophic mutation in a haploid strain.

Each putative haploid strain was screened for mating type. Out of the 148 haploids screened, 75 were of the **a** mating type and 73 were of the alpha mating type. This approximated to a 2:2 ratio as expected for the segregation of the two alleles residing at the *MAT* loci. Strains of the **a** mating type were further screened for temperature sensitivity. 47 *MAT***a** strains displayed a wild type proliferative response at 36°C whilst only 28 displayed temperature sensitivity for proliferation at 36°C. These data deviated from the 2:2 ratio of segregation expected for a single temperature sensitive mutation (see section 2.4, Discussion). Finally, the strains displaying a temperature sensitive phenotype were screened for

auxotrophic requirements (data not shown).

Temperature Shift Analysis of Selected Strains

Three haploid progeny from the cross between MD40/4C and DE/10B were selected for further study. Each strain was of the a mating type, displayed an arrest of proliferation at the restrictive temperature and both a uracil and leucine dependence for growth. The strains TDE/16A and TDE/24C, derived from tetrad dissection and strain RE24 derived from random spore analysis were subjected to temperature shift analysis.

Each of the mutant strains displayed a fall in culture budding index to a value of less than 0.2 after a shift of exponentially growing cells from 25°C to 36°C (Table 2.8). The strain TDE/16A exhibited the largest drop in budding index after the temperature shift. The budding index of the TDE/16A culture fell from a value of 0.77 at the time of the shift to 0.17 after 4 hours of incubation at 36°C. This conditional arrest phenotype was similar to that observed for the primary *dna26-1* mutant JL448.

Identification of the Auxotrophic Mutations in the Temperature Sensitive Mutants

From the genotypes of the parent strains from which they were derived, it was deduced that strains TDE/16A, TDE/24C and RE24 contained the *trp1* and *leu2* genes. For the purposes of cloning work it was important to identify the mutation conferring uracil dependence on TDE/16A. The mutation conferring adenine dependence on this strain was also unidentified. The *ura3* and *ura2* mutations and *ade1* and *ade2* mutations were the possible candidates conferring

STRAIN	TIME AFTER TEMPERATURE SHIFT (Min.)				
	0	60	120	180	240
TDE/16A	0.77	0.60	0.50	0.28	0.17
TDE/24C	0.65	0.42	0.33	0.21	0.13
RE24	0.62	0.54	0.37	0.26	0.15
MD40/4C	0.68	0.73	0.56	0.58	0.61
JL448	0.60	0.27	0.24	0.16	0.12

TABLE 2.8 Culture Budding Indices of Temperature Sensitive Segregants

Three temperature sensitive progeny from the cross between MD40/4C and DE/10B were subjected to temperature shift analysis. Exponentially growing cultures were shifted from 25°C to 36°C at 0min. Culture budding index was measured at hourly intervals for at least 4 hours. The strains MD40/4C and JL448 were included as wild type and temperature sensitive controls respectively.

uracil and adenine dependence respectively. Complementation analyses were therefore carried out using various uracil and adenine auxotrophic mutants of opposite mating type to that of TDE/16A. The temperature sensitive mutants TDE/24C and RE24 were also included in the complementation analyses as alternative candidates for use in subsequent cloning work.

Positive complementation of the *ade1* and *ura1* but not the *ade2* and *ura3* mutations was observed when TDE/16A was mated with the relevant strains (Table 2.9). These results indicated that TDE/16A contained the *ade2* mutation as the sole cause of its adenine dependent phenotype, and the *ura3-251/373* mutation as at least part of the cause of its uracil-requiring phenotype. The unavailability of a non-leaky *MAT*alpha *ura2* strain made it impossible to eliminate the possibility that TDE/16A contained the leaky *ura2* mutation. However later cloning work established that a low copy *URA3* plasmid could fully suppress the uracil dependent phenotype of this strain (see Chapter 4). The genotype of TDE/16A was therefore designated as *MAT***a** *dna26-1 ade2 trp1 leu2-3/112 ura3-251/373*. This strain was employed in all subsequent physiological and molecular biological studies of the *dna26-1* mutation.

PHENOTYPIC CHARACTERIZATION OF A *dna26-1/+* HETEROZYGOTE

The *dna26-1* mutant TDE/16A was crossed with the wild type strain MD40/4C. A single diploid colony was selected, the ploidy of the cells being verified by, i) their inability to produce mating pheromone during a screen employing the halo assay technique and ii) their ability to develop asci on sporulation medium (data not shown). The diploid strain, designated MD/16/D1, was streaked for single

MATa STRAINS	*MAT*alpha STRAINS			
	TD4 (*ura1*)	DBY746 (*ura3*)	SC8 (*ade2*)	SC2 (*ade1*)
TDE/16A	+	-	-	+
RE24	+	-	-	+
TDE/24C	+	-	ND	ND
DBY747 (*ura3*)	+	-	ND	ND
X4003-5B (*ade1*)	ND	ND	+	-
RC631 (*ade2*)	ND	ND	-	+

TABLE 2.9 Complementation Analysis to Identify Auxotrophic Mutations

The data indicate positive or negative complementation of the relevant mutations in diploids produced by crossing the appropriate strains (see Materials and Methods). The three temperature sensitive mutants TDE/16A, TDE/24C and RE24 each contained unidentified mutations conferring uracil and adenine dependence for growth. The strains DBY747, X4003-5B and RC631 were included as control strains carrying the *ura3*, *ade1*, and *ade2* mutations respectively. No appropriate *MAT*alpha *ura2* strain was available to test for the presence or absence of the *ura2* mutation in the temperature sensitive mutants.

ND - Complementation Not Determined.

colonies on YEPD agar. After 4 days of incubation at 36°C, colony formation by MD/16/D1 was comparable to that of the wild type haploid strain MD40/4C. In contrast, no colony formation was observed when the haploid *dna26-1* mutant TDE/16A was incubated under the same conditions.

PHENOTYPIC CHARACTERIZATION OF A *dna26-1 / dna26-1* HOMOZYGOUS DIPLOID

A haploid *MAT*alpha *dna26-1* mutant strain was constructed by firstly crossing TDE/16A (*MAT*a *dna26-1*) with the wild type *MAT*alpha strain SRA/364/47. SRA/364/47 was derived by the author from a wild type strain, A364A described previously (Hartwell, 1967). One diploid colony resulting from the cross was induced to sporulate and the meiotic products isolated by random spore analysis. A strain of the alpha mating type which also displayed a conditional G1 arrest at 36°C was selected and crossed with TDE/16A to produce a diploid designated SRA/TDE/D202.

The diploidy of SRA/TDE/D202 was verified by its ability to sporulate and inability to secrete mating pheromone. SRA/TDE/D202 was streaked for single colonies on YEPD agar and incubated for 4 days at 36°C. No colony formation was observed after this time. The haploid *dna26-1* mutant TDE/16A also failed to form colonies under the same conditions whilst proliferation of the haploid SRA/364/47 at 36°C was similar to its proliferation at 25°C.

COMPLEMENTATION OF THE *srm1-1* MUTATION BY THE *dna26-1* MUTATION

The *MAT*alpha *srm1-1* mutant strain SY1115 (a gift from George Sprague, University of Oregon) was crossed with the *MAT*a *dna26-1* mutant TDE/16A. Diploids were selected and checked for the ability

to sporulate on acetate medium. After incubation on YEPD agar for 4 days at 36°C a diploid containing the *srm1-1* and *dna26-1* mutations displayed a capacity for colony formation that was comparable to that of the temperature resistant *dna26-1 / +* heterozygous diploid control strain MD/16/D1.

TEST FOR INTERACTION BETWEEN THE *dna26-1* MUTATION AND THE *CKS1* GENE

To eliminate the *CKS1* gene as an allele of *DNA26* a transformation experiment was carried out to test for suppression of the *dna26-1* mutation by the plasmid-borne *CKS1* gene. The *dna26-1* mutant TDE/16A was transformed with pJH4-67-13-2 and the cells spread onto selective medium lacking uracil. 5 out of the 10 transformation plates were transferred from 25°C to 36°C after 24hr and colony formation was assessed after 10 days of incubation at either temperature. No transformant colonies were observed on the plates incubated at 36°C whilst 11 colonies were observed on those incubated at 25°C. The plasmid YCpDE8, which had been shown previously to be capable of suppressing the *dna26-1* mutation (see Chapter 4), was used to transform TDE/16A in a parallel experiment. YCpDE8 generated 11 transformants at 25°C and 4 at 36°C.

TEST FOR INTERACTION OF *dna26-1* WITH GENES OF THE *RAS*-cAMP PATHWAY

A series of transformation experiments were carried out to look for possible suppression of the *dna26-1* mutation by overexpression of previously cloned genes. Plasmids carrying the *CDC25*, *CDC35(CYR1)*, *RAS2*, *RAS2^{val19}*, *BCY1* and *TPK1* genes were available and used to transform the *dna26-1* mutant TDE/16A. Transformants were selected by complementation of either the *leu2-3/112* or the *ura3-251/373* mutation

in TDE/16A by the appropriate plasmid marker. Additional selection was then made for the ability of the plasmid to suppress the temperature sensitive phenotype of the *dna26-1* mutant at 36°C.

The results of the transformation experiments are shown in Table 2.10. None of the plasmids carrying genes involved in the cAMP-mediated signal transduction pathway successfully suppressed the temperature sensitive phenotype of the *dna26-1* mutant. Plasmids YCpDE8, YEpDE8 and p801, that had previously been shown to be capable of suppressing the *dna26-1* mutation, (see section 4.3), all permitted the growth of at least some transformants at 36°C whilst the control plasmids YCp50, YEp13 and YEp24 (see Appendix 1) did not.

TABLE 2.10 Transformation of TDE/16A(*dna26-1*) with plasmids carrying Genes Involved in cAMP-Mediated Signal Transduction

The data are for the number of transformants obtained with the relevant plasmids at 25°C and 36°C. Intact cells of TDE/16A were transformed by the lithium acetate method (see section 4.2). Initial selection was for the plasmid marker at 25°C. For each plasmid, 100 transformant colonies were replica-patched to selective medium and proliferation of cells was scored after 4 days of incubation at 25°C or 36°C.

* cAPK, cAMP-dependent protein kinase.

\# Colony formation screened directly at 36°C. 10 transformation plates were initially incubated at 25°C for 30hr. 5 plates were then shifted to 36°C. The number of colonies on the plates at the two respective temperatures was determined after 10 days of incubation.

\## YCpDE8, YEpDE8 and p801 each contain a similar yeast genomic fragment that had been previously shown to be capable of suppressing the *dna26-1* mutation (see section 4.3).

Table 2.10

PLASMID	SELECTIVE MARKER	PROTEIN ENCODED	Nº OF VIABLE COLONIES 25ºC	36ºC	PARENT PLASMID
YEp(RAS2-1) *(RAS2)*	*LEU2*	Ras2 G Protein	100	0	YEp13 (2μm)
YEp(RAS2^{val19}) *(RAS2^{val19})*	*LEU2*	Hyperactivated Ras2 G Protein	100	0	YEp213 (2μm)
pCYR1-2 *(CYR1)*	*URA3*	Adenylyl Cyclase	100	0	YCp50 *(CEN4)*
pBCY1-3 *(BCY1)*	*URA3*	Regulatory Subunit of the cAPK*	100	0	YEp24 (2μm)
YEpTPK1 *(TPK1)*	*LEU2*	Catalytic Subunit of the cAPK	100	0	YEp13 (2μm)
YEp351 *(CDC25)*	*LEU2*	GDP-GTP Exchange Factor	2000#	0	YEp351 (2μm)
pAP5410 *(CDC25)*	*URA3*	'' ''	20#	0	YEp24 (2μm)
YEpDE8##	*URA3*	--	13	11	YEp24
YCpDE8##	*URA3*	--	11	4	YCp50
p801##	*LEU2*	--	20	14	pMA3A (2μm)
YCp50	*URA3*	--	50	0	---
YEp13	*LEU2*	--	50	0	---
YEp24	*URA3*	--	50	0	---

2.4 DISCUSSION

Sporulation Frequency and Spore Viability During Backcrossing

Throughout the backcrossing of *dna26-1* mutants into the wild type strains MD40/4C and A33, a low efficiency of sporulation and spore germination was observed. On average, only 5% of the diploid population at each round of backcrossing produced 3 or 4-spored asci, even after 5-7 days of incubation on sporulation medium. It is likely that the long incubation time required for the production of adequate numbers of tetrads itself contributed to the low frequency of spore germination that was repeatedly observed (Mortimer & Hawthorne, 1969).

It was observed during the random spore analysis of the meiotic products of the cross between MD40/4C and DE/10B that more *MAT***a** ts^+ progeny germinated than *MAT***a** ts^- progeny. The simplest explanation for this is that the mutant *dna26-1* gene product is less than fully functional for its role in mitotic proliferation even at 25°C. As a consequence of this, germination during the random spore analysis may have occurred more slowly in the *dna26-1* mutant spores than in spores that did not contain the mutation. Red colonies were selected after only 3 days at 25°C during this procedure. Fewer of the *dna26-1* spores than wild type spores may have formed red colonies by this time resulting in the unbalanced ratio of ts^+ to ts^- progeny observed. It is also possible that the low frequency of sporulation obtained during the backcrossing experiments was due to a mutation other than the *dna26-1* mutation derived from the original *dna26-1* parent JL448. However, if this had been the case an improvement in sporulation characteristics would have been expected during the repeated backcrossing to MD40/4C as the secondary mutation was

diluted out (under conditions of specific repeated selection for the *dna26-1* mutant phenotype). Little evidence for this was observed although the frequency of asci containing 4 rather than fewer spores increased in the final round of backcrossing as did the frequency of viable spores (data not shown). Frequency of ascus formation *per se* remained constant however at about 5% in each round of backcrossing. It is unlikely that the *dna26-1* mutation is directly involved in the mechanisms of sporulation although from the above observations it seems possible that the mutation may exert some influence over the general vigour of the cell, even in a heterozygous diploid. Construction of the heterozygous diploid MD/16/D1 however revealed that on rich medium at 36°C the *dna26-1* mutation is apparently recessive to its wild type allele, at least for mitotic proliferation.

Segregation of the Temperature Sensitive Marker

The pattern of segregation for the temperature sensitive phenotype during each round of the backcrossing (shown in Tables 2.3, 2.5 and 2.7) suggested that a second temperature sensitive mutation in addition to the *dna26-1* mutation was segregating. A marked deviation from a Mendelian 2:2 ratio of segregation for a single temperature sensitive mutation was observed in the first and second rounds of backcrossing (see Tables 2.3 and 2.5). Interpretation of these data is made tentative however by the low number of asci examined due to the practical difficulties caused by the low sporulation frequencies obtained. The high proportion of asci displaying $3ts^{+}:1ts^{-}$ and $1ts^{+}:3ts^{-}$ ratios of spores is also difficult to interpret.

The first two backcrosses involved mating genetically unrelated

strains. The segregation data obtained from these crosses may therefore have been complicated by the disturbance of complex genetic interactions involving the suppression of temperature sensitive mutations in either or both parents. The backcrossing to strain A33, which is unrelated to MD40/4C, may have aggravated the anomalous segregation data obtained for segregation of the temperature sensitive marker. Furthermore, the exposure of strain JL448 to mutagenic treatment as part of the original screen for temperature sensitive mutants may have also been a factor in generating the complex segregation data that were obtained.

In the final backcross into MD40/4C, 18 out of 22 of the tetrads examined displayed a 2:2 ratio of segregation of temperature sensitive to temperature resistant spores whilst 3 out of the 22 displayed a ratio of $1ts^{+}:3ts^{-}$ spores (Table 2.7). This suggested the segregation of two tightly linked temperature sensitive mutations. The degree of linkage between two such mutations can be calculated by assuming that a single cross-over is the simplest event by which the $1ts^{+}:3ts^{-}$ ratios of spores could have been generated in tetratype asci (Figure 2.1). The standard equation shown below can be used to calculate the genetic map distance between the two putative temperature sensitive mutations (Mortimer & Schild, 1980) :

$$Xp = \frac{100}{2} \quad \frac{TT + 6NPD}{PD+NPD+TT}$$

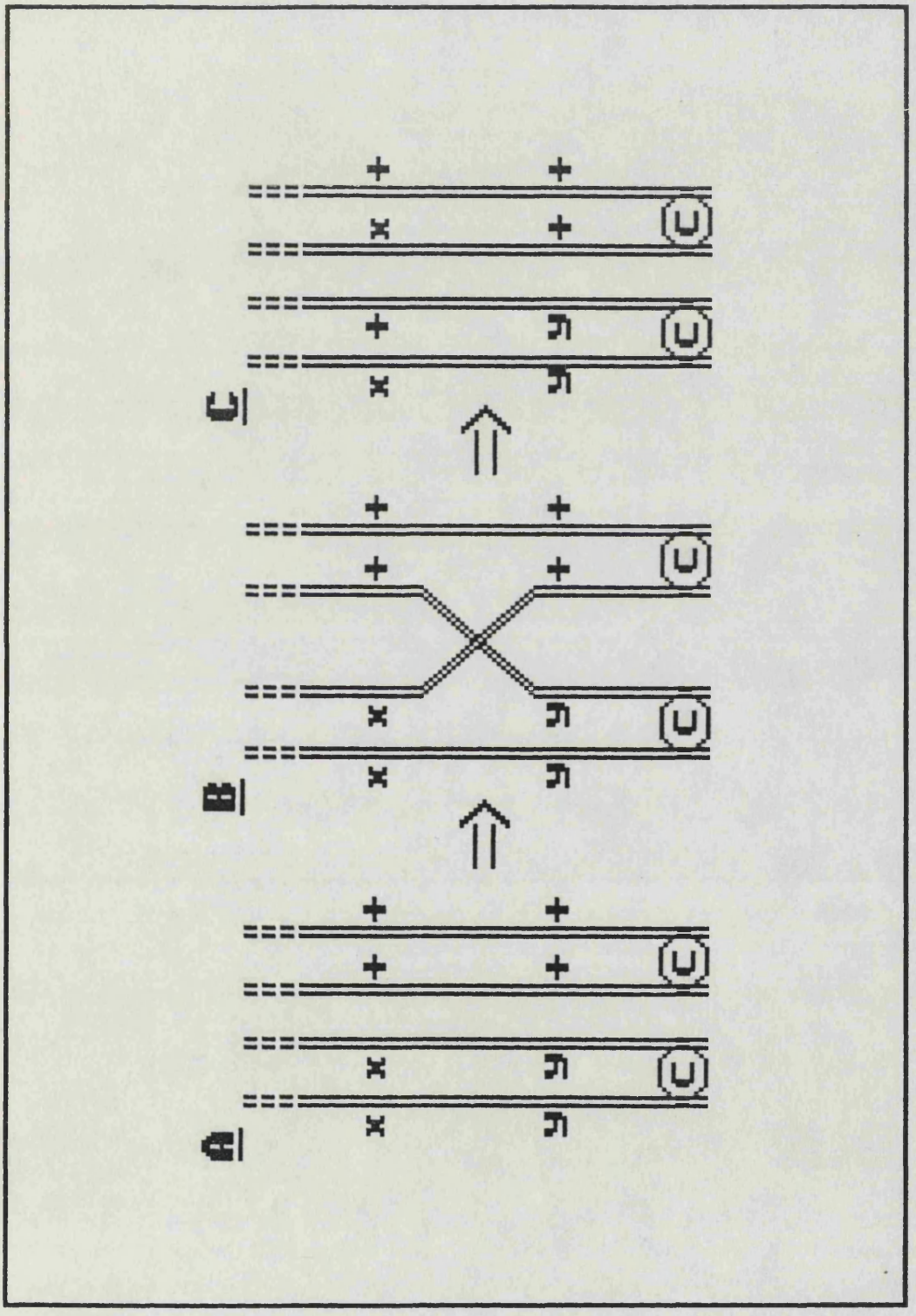
- where Xp is the genetic map distance in centiMorgans, and TT, NPD and PD are the number of tetratype, non-parental ditype and parental ditype asci respectively. 3 apparently tetratype asci and 18 parental ditype asci were obtained from the cross. The theoretical map distance between the two putative temperature sensitive mutations

FIGURE 2.1 Single Cross-over Model for the Production of 1ts^{+}:3ts^{-} Asci

The figure depicts two pairs of homologous chromatids present in a diploid produced by the cross between MD40/4C and DE/10B. Mutations *x* and *y* represent two hypothetical temperature sensitive (ts^{-}) mutations that are closely linked on the same chromosome in DE/10B. Both *x* and *y* have homologous wild type alleles, (+), present on the chromosome derived from MD40/4C. A; 2 pairs of homologous chromatids are present in the diploid strain. B; a single cross-over occurs between two non-sister chromatids in the interval between the *x* and *y* loci. C; 2 recombinant and 2 parental type chromatids are present in the diploid cell after the crossing-over event is completed and form the chromosomes of the haploid progeny resulting from meiotic division and sporulation. To explain the 1ts^{+}:3ts^{-} ratio of spores from this tetratype ascus it is assumed that the presence of either or both of the mutations *x* or *y* confers a ts^{-} phenotype in a haploid cell.

Encircled C - centromere.

Broken lines - sequences centromere distal to *x* and *y*.


A
B
C

is therefore 7.1cM. The single ascus showing a 3ts$^+$:1ts$^-$ ratio of spores was not included in this calculation.

Despite the strict interpretation of the segregation shown above, that two closely linked temperature sensitive mutations are present in the *dna26-1* mutant genome, it is also possible that temperature sensitive mutations were occurring at a low frequency for unexpected reasons during the backcrossing. The uncovering of suppressible temperature sensitive mutations during recombination of yeast genomes has been reported previously (Hanic-Joyce, 1985 - discussed further in section 4.4). Further backcrossing into MD40/4C would have probably more conclusively revealed the nature of the temperature sensitive defect segregating in the above crosses. However, during subsequent cloning work (Chapter 4) the temperature sensitive phenotype of the mutant TDE/16A was assumed to be caused by a single mutation rather than by the interactive effect of two linked mutations.

The Suitability of the *dna26-1* Mutant TDE/16A for Cloning Procedures

The mutant strain TDE/16A, derived from the cross between MD40/4C and DE/10B, displayed a *cdc* phenotype similar to that of the primary *dna26-1* mutant JL448. During the temperature shift experiment (Table 2.8), more than 80% of the cells in the TDE/16A culture displayed an unbudded morphology after incubation for 5 hours at 36°C. The kinetics of G1 arrest were however slower for TDE/16A than for JL448. The reason for this may be that JL448 contains other temperature sensitive mutations in addition to the *dna26-1* mutation which synergistically enhance the temperature sensitive defect caused by the *dna26-1* mutation in this strain. Alternatively, the different

kinetics of arrest may be merely a reflection of the different genetic backgrounds in which the *dna26-1* mutation resides in the two mutant strains.

Study of the mating response in yeast often requires the use of commercially available alpha factor. Moreover, alpha factor is commonly used in cell cycle studies as a general cell cycle inhibitor which synchronously arrests cells in G1. A further basis for the selection of TDE/16A for further study was therefore its a mating type, rendering it amenable to cell cycle studies involving the use of alpha factor. TDE/16A was also selected for its four auxotrophic mutations *ade2, trp1, leu2-3/112* and *ura3-251/373*. An *ade2* mutation causes the cellular accumulation of a red pigment caused by a defect in the adenine biosynthetic pathway. This pigment formation therefore provided a means by which colonies of TDE/16A might be discriminated from contaminating yeasts. Complementation of the recessive *ade2* mutation also provided a potential means to confirm diploidization when mating TDE/16A with an *ADE2*$^+$ strain during strain constructions. The *LEU2, URA3* and *TRP1* genes are commonly employed as prototrophic markers for selection of yeast plasmid vectors (Parent *et al.,* 1985). The *leu2, ura3* and *trp1* mutations in TDE/16A therefore provided versatility for the use of the mutant in molecular work as a transformation host strain.

The Effect of Cell Type on the *dna26-1* Mutation

A background of heterozygosity at the mating type locus has been reported to cause suppression of certain mutations that in haploid strains cause conditional arrest of the cell cycle at START (Shuster, 1982a; Clark & Sprague, 1989; De Barros Lopes, 1990). Nevertheless

the *MAT*a/*MAT*alpha diploid strain SRA/TDE/D202 homozygous for the *dna26-1* mutation, failed to form colonies when incubated at the restrictive temperature. Furthermore, SRA/TDE/D202 exhibited an arrest of the cell cycle in G1 during a temperature shift analysis in liquid medium (data not shown). This behaviour contrasts with that observed in mutant diploids homozygous for the *cdc36*, *cdc39* or *srm1-1* START mutations. Neither of the latter temperature sensitive mutations causes the usual conditional G1 arrest when homozygous in a diploid cell that is heterozygous at the mating type locus. These observations suggest that the *dna26-1* mutation is not directly involved in the mating pheromone signal transduction pathway and are consistent with the positive complementation observed between the *dna26-1* and *srm1-1* mutations. Also consistent with the above was the equal number of temperature sensitive *MAT*a and *MAT*alpha segregants obtained from the cross between MD 40/4C and DE/10B, suggesting that the *dna26-1* mutation was not influenced by haploid cell type. In addition, the observation that the temperature sensitive mutation was not linked to the *MAT* locus provided evidence that the *dna26-1* mutation is not situated on chromosome III (Mortimer *et al*; 1989).

The Cloned *CKS1* Gene does not Suppress the *dna26-1* Mutation

The *CKS1* gene was cloned as a dosage suppressor of the START mutation *cdc28* (Hadwiger *et al.*, 1989a). The dosage suppression experiment using the high copy plasmid pJH4-67-13-2(*CKS1*) to transform TDE/16A generated no transformants at the restrictive temperature. Although the total number of transformants obtained in this experiment was low it provided evidence that the *DNA26* and *CKS1* genes are non-allelic. The failure of pJH4-67-13-2 to suppress the *dna26-1* mutation at high

copy number also supported the conclusion from previous classical complementation analysis that *DNA26* and *CDC28* are different genes (Davies, 1985).

Genes of the *RAS*-cAMP Signalling Pathway do not Suppress the *dna26-1* Mutation

The cloned *CDC25*, *CDC35*, *RAS2*, *BCY1* and *TPK1* genes were individually unable, when expressed in TDE/16A on autonomous plasmids, to suppress the temperature sensitive growth defect caused by the *dna26-1* mutation. It is therefore unlikely that the *dna26-1* mutation is allelic to any of these genes. Moreover, the results of these transformation experiments argue against the *dna26-1* mutant phenotype being directly due to a defect in *RAS*/cAMP-mediated nutrient signalling.

CHAPTER 3 : PHYSIOLOGICAL CHARACTERIZATION OF THE *dna26-1* MUTANT TDE/16A

3.1 INTRODUCTION

CLASSIFICATION OF THE *dna26-1* MUTATION

The *dna26-1* mutation was originally identified in a screen for mutants that displayed a temperature sensitive defect for DNA synthesis whilst continuing to synthesize protein (Dumas *et al.*, 1982). This screen also inherently favoured the isolation of Class I START mutants due to the requirement for continued growth at the restrictive temperature. Initial physiological studies on the primary *dna26-1* mutant strain JL448, that was isolated from the above screen, supported its classification as a Class I START mutant. JL448 was found to, i) undergo cell cycle arrest at a point interdependent with the point of arrest of mating pheromone-treated cells (White, 1984; Davies, 1985), ii) retain a high conjugational competency at 34°C (Davies, 1985), and iii) continue uptake of a radiolabelled amino acid at 36°C (Green, 1986). JL448 was also used to construct a strain that was homozygous for *dna26-1* and heterozygous at the mating type locus. No suppression of the *dna26-1* mutation was observed in this strain (Green, 1986). The above physiological characteristics of JL448 coupled with the observation that it did not develop a shmoo morphology at the restrictive temperature suggested that it was a Class I START mutant (see section 1.8). Its mutant phenotype is similar to that of the Class I mutants carrying mutations in the *CDC61, CDC62, CDC63* and *CDC68* genes.

The *dna26-1* mutation was purified by backcrossing JL448 into a wild type genetic background (see Chapter 2). One major reason for this was to eliminate any secondary mutations that may have been generated in JL448 during mutagenic treatment with ethyl methane sulphonate.

It was considered that such mutations might interact with the *dna26-1* mutation affecting the phenotype of the *dna26-1* mutant. It was also considered necessary therefore to recharacterize the effect of the *dna26-1* mutation on cellular physiology in the mutant strain TDE/16A that was derived from the backcrossing procedure. A sequential physiological characterization of TDE/16A was carried out with the nature of consecutive analyses being decided by the results of those preceeding. An introduction to the aspects of physiology studied in TDE/16A and the methods of analysis employed is given in the following paragraphs.

Kinetics of G1 Arrest

Conditional lethal *cdc* mutations may encode mutant gene products that are either temperature labile or temperature sensitive for synthesis (Pringle, 1978). Determination of the speed of cell cycle arrest after the shift of a *cdc* mutant to restrictive conditions can give an insight into the nature of the genetic lesion present. Mutant gene products that are temperature sensitive for synthesis can be expected to cause a gradual arrest of the cell cycle. The kinetics will however be dependent on the rate of turnover of the gene product and the execution point of gene function. The expectation that a temperature-labile gene product will display comparatively tighter arrest is complicated by the allele-specific phenomenon of leakiness. Leakiness of a conditional mutation can be revealed however both from data describing the kinetics of cell cycle arrest and from observation of the terminal phenotype of a mutant culture (Hartwell, 1974). It was therefore considered that determining the kinetics of cell cycle arrest and terminal phenotype of a TDE/16A mutant culture would provide an informative first step in the physiological

characterization of this strain.

Mating Competency

Conjugation between two haploid cells of opposite mating type can only occur efficiently during the G1 phase of the cell cycle (Reid & Hartwell, 1977). Reciprocal pheromone signalling between haploid cells maximizes mating efficiency by synchronizing the cell cycles of the mating partners at START (Wilkinson & Pringle, 1974; Hereford & Hartwell, 1974). Assessment of the capacity of a conditional START mutant to conjugate under restrictive conditions can provide an indication of the mechanism underlying the cell cycle defect observed in that mutant. Several START mutations have been specifically identified on the criterion of a high competency for mating under restrictive conditions (Reed, 1980). Others have been identified by their ability to suppress mutations involved in the pheromone signal transduction pathway (Reed *et al.*, 1988; Jahng *et al.*, 1988; Clark & Sprague, 1989). These screening procedures have identified mutations that are directly involved in mechanisms of pheromone dependent cell cycle control at START. Other mating competent Class I START mutations have however been identified that apparently do not have a direct role in the mating response (Bedard *et al.*, 1981; Prendergast *et al.*, 1990a).

Class II START mutants invariably display a poor competency for conjugation under restrictive conditions (Reid & Hartwell, 1977; Bedard *et al.*, 1981). This inability to mate during START arrest is probably a consequence of the growth defect displayed by these mutants.

The mating ability of the *dna26-1* mutant TDE/16A was therefore

investigated as part of a further physiological characterization of the effect of the *dna26-1* mutation.

Macromolecular Metabolism

START mutants can be classified on the basis of their biosynthetic activity during conditional START arrest (Reed, 1980). Class I START mutants maintain the continuous processes of protein and ribonucleic acid (RNA) synthesis under restrictive conditions despite the arrest of cell cycle progression in G1 and arrest of DNA synthesis. This has led to the suggestion that Class I START mutations such as *cdc36, cdc37, cdc39, cdc28, cdc62, cdc63, cdc68* and *mak16* cause a defect in genes that have an integral function in the regulatory mechanisms controlling cellular proliferation.

In contrast to Class I START mutants, Class II mutants containing mutations such as *cdc35(cyr1)* (Boutelet *et al.*, 1985; Matsumoto *et al.*, 1983), *cdc25* (Martegani *et al.*, 1984), *cdc60, cdc64, gcd1* and *ils1* display a substantial decline in the rate of macromolecule synthesis during START arrest. This characteristic suggests that the biosynthetic defect evident in Class II mutants prevents performance of START more indirectly by preventing cells from attaining the minimum cell size required for START execution (Johnston *et al.*, 1977a). This argument is complicated however by the physiological effects of the Class II *prt1-1* mutation which causes a defect in translation initiation. Under less severe restrictive conditions the *prt1-1* mutation can also cause a Class I-type START arrest despite a protein synthetic capacity that is apparently sufficient for attainment of the critical size for cell cycle initiation (Hanic-Joyce *et al.*, 1987a).

To further classify the *dna26-1* mutation, its conditional effect on protein synthesis was investigated in the mutant TDE/16A.

Thermotolerance of arrested START mutants

Stationary phase cells exhibit a number of characteristic properties, including the accumulation of storage carbohydrates (Lillie & Pringle, 1980), resistance to cell wall-degrading enzymes (Deutch & Parry, 1974) and resistance to killing by heat (Walton *et al.*, 1979; Schenberg-Frascino & Moustacchi, 1972; Parry *et al.*, 1976).

It has also been demonstrated that Class II START mutants, which arrest with stationary phase-like characteristics, accumulate a range of so-called GO proteins that are also seen to accumulate in nutritionally starved cells (Iida & Yahara, 1984b; Iida & Yahara, 1984c; Boucherie, 1985) and of which some are believed to be heat shock proteins (Lindquist & Craig, 1988). Such START mutants simultaneously acquire a durable thermotolerance to high non-physiological temperatures (Plesset *et al.*, 1987; Shin *et al.*, 1987), although the correlation between the accumulation of GO proteins and the acquisition of thermotolerance is somewhat unclear (see Barnes *et al.*, 1990; Sanchez & Lindquist, 1990).

Incubation of Class I START mutants at a restrictive temperature of 36°C induces thermotolerance (Plesset *et al.*, 1987). This response is also shown by wild type strains exposed to mild heat shock at 36°C (Plesset *et al.*, 1987; McAlister & Finkelstein, 1980). In contrast to Class I START mutants, wild type cells display only a transient G1 arrest when exposed to a temperature of 36°C (Johnston & Singer, 1980). Furthermore the induced thermotolerance of both Class I START mutants and wild type strains is only transient (Plesset *et al.*,

1982; 1987). This response contrasts with the durable thermotolerance induced in temperature arrested Class II START mutants.

The *dna26-1* mutant TDE/16A was therefore examined for its resistance to killing by elevated temperature in order to further classify its mode of cell cycle arrest under restrictive conditions.

3.2 MATERIALS AND METHODS

STRAINS

The strains of *S. cerevisiae* used in the physiological studies are listed in Table 3.1.

MEDIA

The YEPD and SD media used in the following work are described in section 2.2.

DETERMINATION OF CULTURE DENSITIES AND BUDDING INDEX

Electrozone Celloscope Particle Counter

A 1ml sample of a yeast culture was suspended in 9ml of a solution of 0.15M NaCl / 3.7% (v/v) formaldehyde. The cells were lightly sonicated for 10secs and the sample was made up to 50ml with an ultra-filtered (pore size 0.2μm) solution of 0.1%(w/v) sodium azide / 0.9%(w/v) sodium chloride. Five readings for cell number were made using an electronic particle counter (Particle Data Inc.) which measures 200μl of diluted culture.

Haemocytometer Counts

An "Improved Neubauer" haemocytometer was used to estimate the cell density in yeast cultures. 250μl aliquots of a yeast culture were lightly sonicated and 7μl immediately removed for cell counting. The number of cells/ml was determined by multiplying the total number of cells counted within the complete haemocytometer grid by a factor of 10000.

Strain	Mating Type	Genotype	Source
TDE/16A	a	*dna26-1 ade2 trp1 leu2-3/112 ura2-251/373*	D.E.
TDE/6B	a	*ade2 trp1 leu2-3/112 his3-11/15* lys-	D.E.
MD40/4C	alpha	*trp1 leu2-3/112 his3-11/15 ura2*	M.T.
SM6	a	*cdc28-6 tyr1 met8-1 arg1*	B.C.
BR214-4a	a	*cdc35-1 ade1 his7 arg4-1 trp1 ura1*	J.D.
BR35/7A	a	*cdc35-1 his7 met2 tyr1*	D.E.
SR661-2	a	*cdc36-1 ura1 trp1 tyr1 gal1*	S.R.

TABLE 3.1 Yeast Strains Employed in Physiological Experiments

The genotypes of the strains used during the physiological characterization of the *dna26-1* mutant are shown as well as the source from which each was obtained. D.E., constructed by author; M.T., Mick Tuite; J.D., Dick Dickinson; B.C., Bruce Carter; S.R., Steve Reed.

Budding Index

Culture budding index was measured as described in section 2.2

TEMPERATURE SHIFT EXPERIMENTS

A single colony was used to inoculate 7ml of YEPD. The culture was incubated at 24°C in a shaking water bath for approximately 30hr until a cell density of $2X10^8$/ml had been reached. This starter culure was used to inoculate 75ml of YEPD. The culture was grown overnight to a density of approximately $1X10^6$/ml in a shaking water bath (set at 106rpm) at 24°C. Before the shift to 37°C the culture was sampled at hourly intervals for the determination of budding index and cell number. When the cell number was increasing at an exponential rate the culture was shifted to a 37°C shaking water bath (106rpm) and samples for budding index and culture density determination were taken at hourly intervals for at least another 5 hours.

PHOTOGRAPHY OF YEAST CELLS

Yeast cells were photographed after fixing in formaldehyde and brief sonication. Cell morphology was examined under phase contrast X40 magnification using an Olympus BH-2 light microscope. The cells were photographed using an Olympus OM10 camera.

DETERMINATION OF MATING COMPETENCY

Quantitative mating assays were carried out essentially as described by Reid & Hartwell (1977). Mating of a temperature sensitive (ts^-) mutant with a temperature resistant (ts^+) strain of opposite mating

type was carried out under three parallel sets of conditions. The first experiment was designed to estimate the mating competency of ts^- mutants under permissive conditions and was termed asynchronous 25°C mating. Cultures of each strain were grown at 25°C to a density of 1-5X10^6/ml in a shaking water bath. 1.5ml aliquots were removed from each culture and mixed by filtering on to a Whatman 2.5cm-diameter nitrocellulose disc (pore size 0.45μm). The filter was placed onto a YEPD feeder plate and incubated at 25°C for 4hr. The cells were resuspended in 3ml of 1M sorbitol by vortexing for 20secs, lightly sonicated (7secs) and serially diluted in 1M sorbitol. 100μl aliquots of the appropriate dilutions were spread onto YEPD plates for cell viability determination and onto supplemented SD plates for diploid selection and quantification. Colony counts were made after 3 days of incubation at 25°C.

The second experiment was designed to assess non-specific depression of mating competency in the ts^- mutants caused by the general sickness induced by incubation of cells at 37°C. This experiment was termed synchronous 25°C mating. The ts^- mutant was preincubated at 37°C for 4 hr prior to mating. 1.5ml aliquots of the ts^- and ts^+ cultures were then mixed on a nitrocellulose disc, placed on a YEPD plate and mating allowed to occur at 25°C for 4hr. Diploidization and cell viability were determined as described for the asynchronous, 25°C experiment.

The third experiment was designed to ascertain the nature of the START arrest seen in the ts^- mutants studied and was termed asynchronous 34°C mating. The ts^- mating partner was preincubated at 37°C for 4 hours prior to mating. This caused arrest of the cell cycle of the ts^- START mutants in G1. 1.5ml aliquots of the ts^- and

ts$^+$ cultures were then mixed by filtering onto a nitrocellulose disc. The disc was placed on a YEPD feeder plate that had been prewarmed to 34°C and incubated at 34°C for 4hr. Resuspension, dilution and quantitation of viable and diploid cells was carried out as described above.

INCORPORATION OF RADIOLABELLED AMINO ACIDS

The incorporation of radiolabelled amino acids into cellular protein was estimated essentially by the method of Hanic-Joyce *et al.*, (1987a). Two experiments were carried out, the first using [U^{14}C]-labelled arginine and the second using a [U^{14}C]-labelled protein hydrolysate.

Incorporation of [U^{14}C]-arginine

The [U^{14}C]arginine monohydrochloride (Amersham International plc.) had a specific activity of 345 mCi/mmol and a radioactive concentration of 50μCi/ml. Cells were grown to a density of 1-5X10^6/ml in supplemented SD medium at 25°C in a shaking water bath set at 106rpm. Cell number and budding index were monitored at hourly intervals. When the cell number was increasing exponentially the culture was shifted to 36°C in a shaking water bath set at 106rpm. A 1ml culture sample was immediately transferred to a test tube that had been prewarmed at 36°C and which contained 10μl of [U^{14}C]-arginine (0.5μCi (0.0185MBq)). Label incorporation was allowed for 15min at 36°C with shaking. The sample was then transferred to an ice bath and an equal volume of an ice cold solution of 10%(w/v) trichloroacetic acid (TCA) / 20μg/ml arginine added and the suspension mixed by briefly shaking the tube. The

sample was left on ice for 1hr. Acid precipitable material was collected onto a cellulose acetate filter (Sartorius), of pore size 0.25μm, by vacuum filtration. The filter was washed with a total of 50ml of 5%(w/v) TCA / 20μg/ml arginine in 5 consecutive washes and finally transferred to 5ml of Optiphase "Safe" liquid scintillant (LKB). The level of radiolabel incorporation was determined in a Packard 3255 scintillation counter set for a 7min counting period per vial. Sampling of the yeast culture was continued for 4-5hr after the temperature shift. Background levels of radioactivity were estimated by fixing cells prior to label incorporation and subsequently measuring the radioactivity of acid precipitable material.

Uptake of [U^{14}C]-labelled Protein Hydrolysate

The [U^{14}C]-protein hydrolysate employed had a specific activity of 57mCi/mATOM and a radioactive concentration of 250μCi/ml. The procedure for measuring label incorporation into cellular protein was identical to that for [U^{14}C]-arginine incorporation with just a few modifications. The permissive temperature used for growth of ts$^-$ strains in this experiment was 24°C and the restrictive temperature used was 36.5°C. These growth temperatures were employed in an attempt to compensate for the low rate of label incorporation previously observed for strain TDE/16A (*dna26-1*) at the permissive temperature compared with other strains and any leakiness of the *dna26-1* mutation at 36°C. The amount of labelled hydrolysate added to the reaction tube for TDE/16A culture samples was 4-fold greater (40μl/10μCi) than that for the control strains used. The duration of label incorporation was reduced in this second experiment from 15 to 10min.

THERMOTOLERANCE ASSAY

The measurement of heat shock resistance was carried out as described by Plesset *et al.*, (1987). Yeast cells were grown in YEPD medium in a 25°C water bath shaking at 106rpm. Samples for budding index and cell counts were taken at regular intervals until the time of the heat shock. Cell counts were made using a haemocytometer. When the cell number in a culture was increasing exponentially at a culture density of $1-5X10^6$/ml, the culture was shifted to a 36.5°C water bath shaking at 106rpm and incubated for a further 5hr. Two 200µl aliquots of the culture were then removed and each placed in a sterile 1.5ml microfuge tube. One tube was immediately placed in an ice-water bath and chilled for 3min. The other tube was immediately heat shocked for 10min in a covered water bath heated to a constant temperature of 52°C, then cooled in an ice-water bath for 3min. The culture sample in each microfuge tube was lightly sonicated (7secs) and serially diluted in 1M sorbitol. 100µl aliquots of the appropriate dilutions were spread onto YEPD plates. Viable cell counts were determined after 4-5 days of incubation at 25°C.

3.3 RESULTS

THE KINETICS OF CELL CYCLE ARREST IN THE *dna26-1* MUTANT TDE/16A

The conditional effect of the *dna26-1* mutation on cell cycle progression was investigated in strain TDE/16A by temperature shift analysis. At the permissive temperature (24°C), the cells in the TDE/16A culture were found at all stages of the cell cycle (Plate 3.1A). In contrast, after a 5hr incubation at the restrictive temperature (37°C) the majority of the *dna26-1* mutant cells displayed an unbudded morphology. This morphology is typical of cells that have arrested in the G1 phase of the *S. cerevisiae* cell cycle (Plate 3.1B).

After the temperature shift from 24°C to 37°C the percentage of unbudded cells in the mutant population rose to greater than 80% over an incubation period of 5hr at the restrictive temperature (Figure 3.1A). Although a complete cessation of proliferation was not observed, a large decrease in the rate of cell number increase occurred within one generation time after the temperature shift (Figure 3.1B). The doubling time of the mutant culture increased from 3hr at 24°C (specific growth rate (μ) = 0.23.hr^{-1}) to 15hr at 37°C (μ = 0.046.hr^{-1} - specific growth rate cited for comparative purposes only as the value of μ obtained at the restrictive temperature was probably not representative of balanced growth).

QUANTITATIVE MATING ASSAYS

The restrictive temperature used to cause cell cycle arrest of START mutants during the mating assays was 37°C as this was the temperature routinely used to cause cell cycle arrest of the *dna26-1*

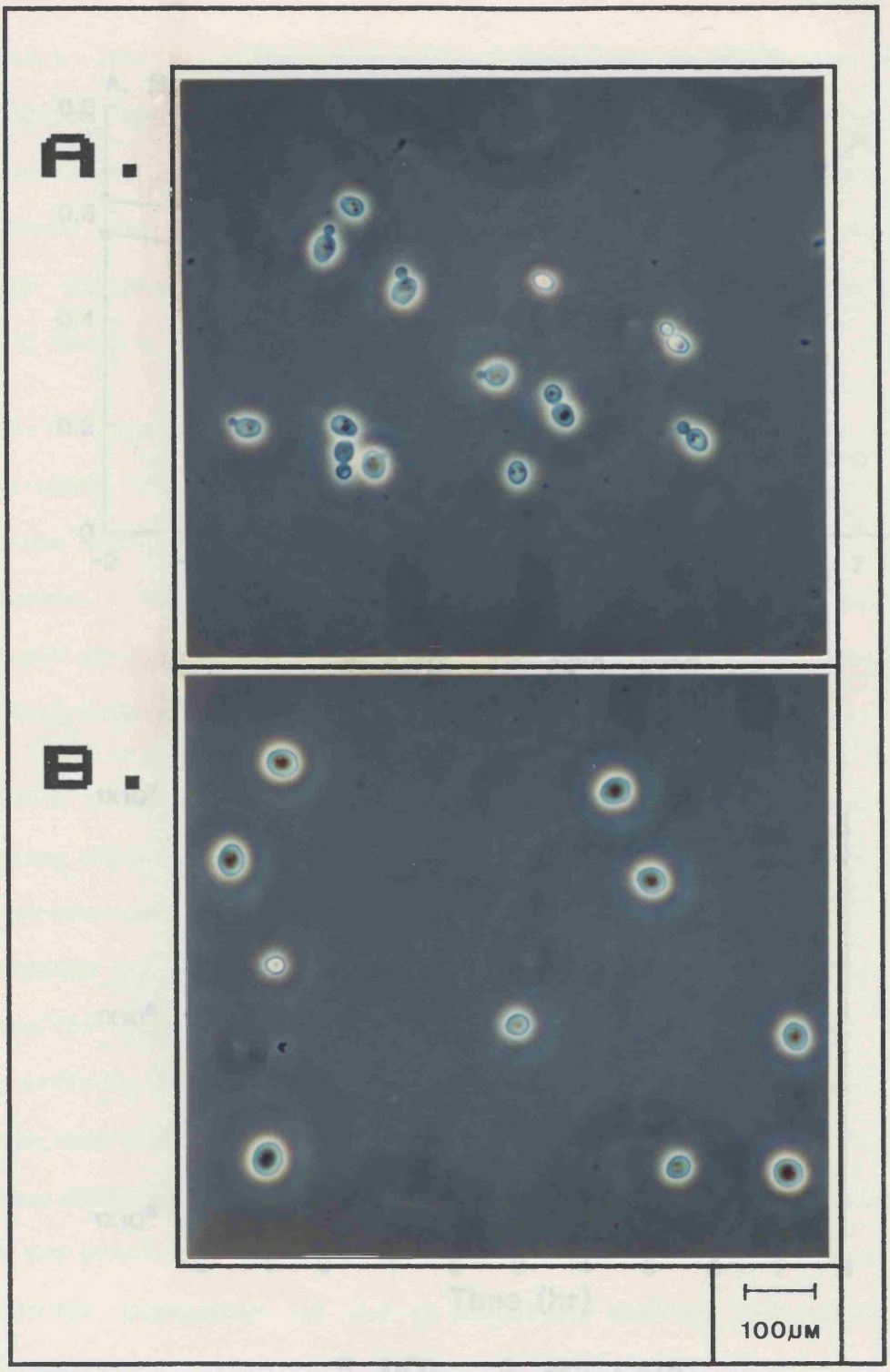


PLATE 3.1 Mutant Cell Morphology at 25°C and 36°C

Cells of the *dna26-1* mutant TDE/16A were grown to a cell density of $6X10^6$/ml in YEPD at 25°C. The cells were then, A, allowed to continue exponential growth to a cell density of $1X10^7$/ml, or B, shifted to 37°C and incubated for a further 5hr. The cells were fixed and sonicated before visualization by phase contrast microscopy (magnification X40).

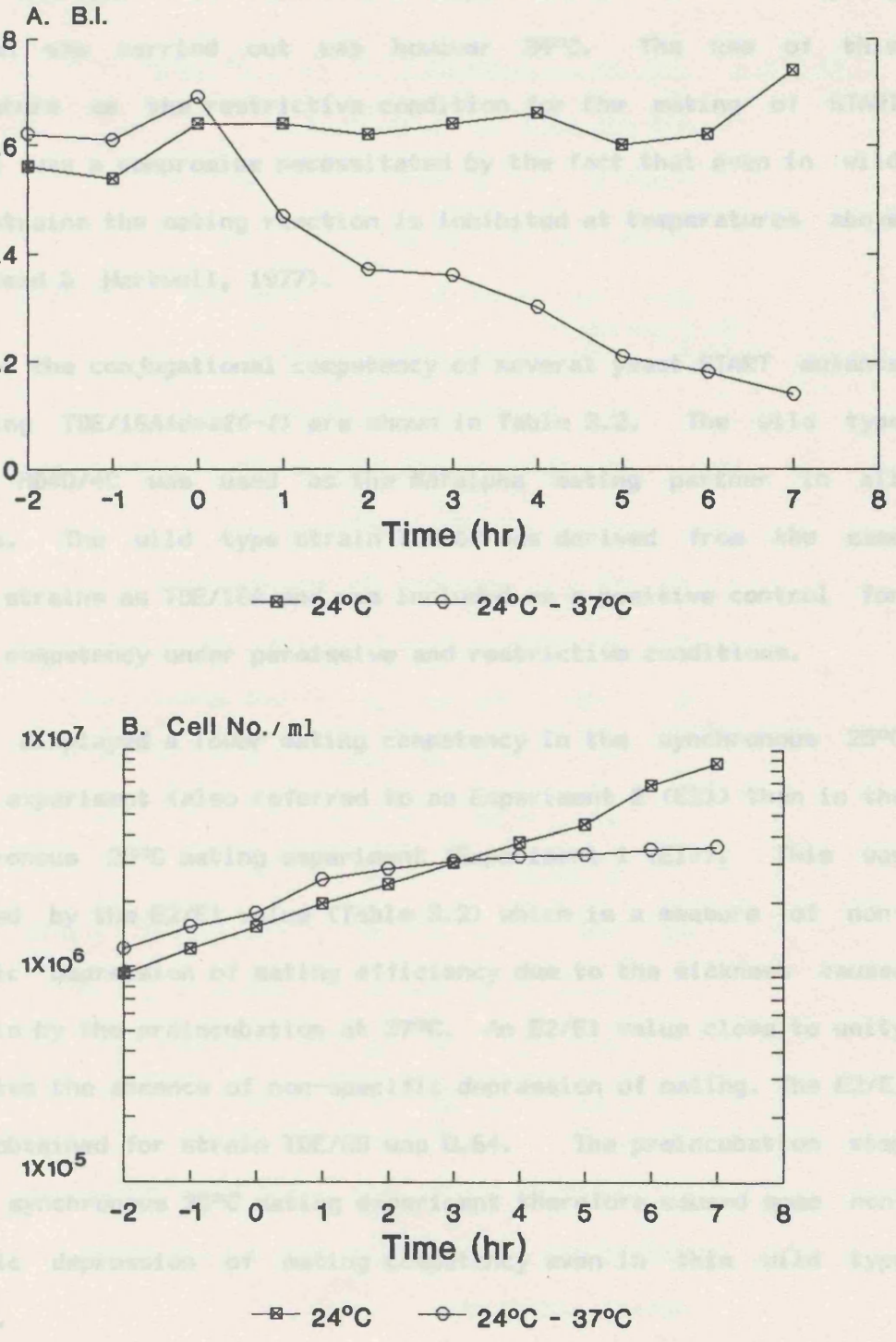


FIGURE 3.1 G1 Arrest Phenotype of TDE/16A

The parameters of budding index (B.I.), A, and cell number, B, were monitored for two parallel cultures of the *dna26-1* mutant TDE/16A grown in YEPD at 24°C in a shaking water bath. At time t=0, one of the cultures was transferred to a 37°C shaking water bath.

mutant TDE/16A. The restrictive temperature at which the mating reaction was carried out was however 34°C. The use of this temperature as the restrictive condition for the mating of START mutants was a compromise necessitated by the fact that even in wild type strains the mating reaction is inhibited at temperatures above 34°C (Reid & Hartwell, 1977).

Data for the conjugational competency of several yeast START mutants including TDE/16A(*dna26-1*) are shown in Table 3.2. The wild type strain MD40/4C was used as the *MAT*alpha mating partner in all crosses. The wild type strain TDE/6B was derived from the same parent strains as TDE/16A and was included as a positive control for mating competency under permissive and restrictive conditions.

TDE/6B displayed a lower mating competency in the synchronous 25°C mating experiment (also referred to as Experiment 2 (E2)) than in the asynchronous 25°C mating experiment (Experiment 1 (E1)). This was revealed by the E2/E1 value (Table 3.2) which is a measure of non-specific depression of mating efficiency due to the sickness caused in cells by the preincubation at 37°C. An E2/E1 value close to unity indicates the absence of non-specific depression of mating. The E2/E1 value obtained for strain TDE/6B was 0.64. The preincubation step in the synchronous 25°C mating experiment therefore caused some non-specific depression of mating competency even in this wild type strain.

A further depression of mating competency was observed in TDE/6B in the synchronous 34°C mating experiment (Experiment 3 (E3)). This was revealed by the E3/E2 value of 0.64 obtained (Table 3.2). The E3/E2 value gives an estimation of the true mating competency of

TABLE 3.2 The Mating Competency of START Mutants Under Permissive and Non-Permissive Conditions

Quantitative mating assays were carried out for the *dna26-1* mutant TDE/16A and a range of control strains as described in section 3.2, Materials and Methods.

* Expt. - Experiment number.

E1 - asynchronous 25°C mating.

E2 - synchronous 25°C mating.

E3 - synchronous 34°C mating.

** VCC(v) - viable cell counts (determined on YEPD)

~ DIPLOIDS - diploid colony count (determined on SD)

\# E2/E1 - the quotient of the mating competency value (d/v) in E2 divided by the mating competency value (d/v) in E1; (non-specific mating depression due to sickness).

\#\# E3/E2 - the quotient of the mating competency value (d/v) in E3 divided by the mating competency value (d/v) in E2; (specific effect of the relevant mutation on mating competency).

A comparison of the values for E3/E2 and E2/E1 gives an indication of the true effect of a START mutation on mating competency.

Table 3.2

		CELL COUNTS		MATING EFFICIENCY		
STRAIN	EXPT.*	VCC(v)**	DIPLOIDS(d)~	(d/v)	E2/E1#	E3/E2##
TDE/6B (*CDC+*)	E1	$8.30X10^5$	$1.40X10^5$	0.1680		
	E2	$1.10X10^6$	$1.18X10^5$	0.1070	0.64	0.64
	E3	$2.47X10^6$	$1.71X10^5$	0.0690		
SR661-2 (*cdc36*)	E1	$2.81X10^6$	$9.70X10^5$	0.3440		
	E2	$2.87X10^6$	$5.80X10^4$	0.0200	0.06	0.55
	E3	$2.84X10^6$	$3.10X10^4$	0.0110		
BR214-4A (*cdc35*)	E1	$2.46X10^6$	$1.90X10^5$	0.0770		
	E2	$2.23X10^6$	$6.16X10^4$	0.0270	0.35	0.0074
	E3	$5.50X10^6$	$1.09X10^3$	0.0002		
TDE/16A (*dna26*)	E1	$6.80X10^5$	$1.43X10^5$	0.2100		
	E2	$6.60X10^5$	$2.00X10^4$	0.0300	0.14	0.07
	E3	$5.80X10^6$	$1.30X10^4$	0.0022		

mutant cells synchronized at a point in the cell cycle by a *cdc* mutation. For a wild type strain such as TDE/6B that does not undergo cell cycle-arrest at 37°C, the E3/E2 value merely gives an estimation of mating competency at 34°C. An E3/E2 value close to unity is an indication of a high efficiency of mating at 34°C. The data obtained for the wild type strain TDE/6B indicated that the mating reaction was less efficient at 34°C than at 25°C in this strain. This was probably due to the fact that 34°C is higher than the optimal temperature for the growth of *S. cerevisiae*.

In contrast to the START mutants used in these experiments, the cells of the wild type strain TDE/6B were not arrested at START by the preincubation at 37°C. Therefore at the time of mixing with the *MAT*alpha mating strain the TDE/6B cell population consisted of cells at all stages of the cell cycle. Mating can only occur at START (Reid & Hartwell, 1977) and therefore some of the 4hr period allowed for the mating reaction in the assay involving TDE/6B was taken up by the time required for the wild type cells to complete their current cell cycle and arrest at START in response to mating pheromone. The mating competency of the unsynchronized TDE/6B culture as judged by this assay may therefore have been an underestimation of the mating competency of this strain compared with the START mutants that were already synchronized in G1 prior to the mating assay.

The Class I START mutant SR661-2(*cdc36-16*) was included in these experiments as a positive control for mating competency at the restrictive temperature. The *cdc36* mutant experienced a marked non-specific depression of mating competency in the synchronous 25°C experiment (E2) which contrasted with its high mating efficiency in the asynchronous 25°C experiment (E1). This was revealed by the

E2/E1 value for this strain of 0.06. Despite this non-specific depression of mating, the difference in mating competency under the conditions of the synchronous 25°C and synchronous 34°C mating experiments was less marked. This was indicated by an E3/E2 value of 0.55 for this strain. This revealed that the *cdc36-16* mutation in SR661-2 permitted efficient mating during cell cycle arrest at the restrictive temperature. This result was as expected for a Class I START mutant originally identified by its mating ability under restrictive conditions (Reed, 1980).

The *cdc35-1* mutant BR214-4a was included in the quantitative mating assays as a negative control for mating under restrictive conditions. Non-specific depression of mating by preincubation at 37°C was again observed in this mutant by comparing its mating efficiency in the asynchronous 25°C and synchronous 25°C experiments. The E2/E1 value of 0.35 obtained for this strain revealed that it was more resilient to the preincubation step at 37°C than the *cdc36-16* mutant. Comparison of the mating efficiecy of the *cdc35-1* mutant in the synchronous 25°C and synchronous 34°C experiments however revealed a marked depression of mating competency at 34°C that was specifically caused by the *cdc35-1* mutation (E3/E2 = 0.074). This result was similar to the previously reported mating characteristics of a temperature sensitive *cyr1-2^{ts}* mutant (Matsumoto *et al.*, 1983).

The *dna26-1* mutant TDE/16A displayed a non-specific depression of mating efficiency in the synchronous 25°C mating experiment which contrasted with its high mating efficiency in the asynchronous 25°C mating experiment. The degree of non-specific inhibition of mating was more severe than that observed in the *cdc35* mutant but less severe than that observed in the *cdc36* mutant (E2/E1 = 0.17). This

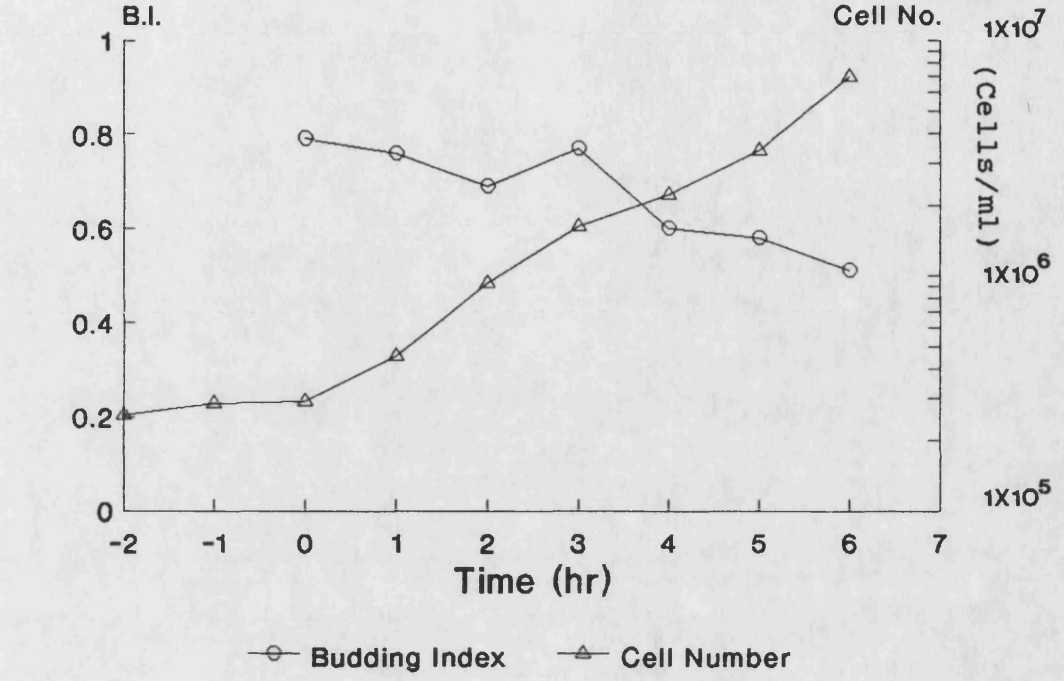
again indicated that a degree of sickness was induced in the cells of the *dna26-1* mutant by preincubation at 37°C. Furthermore, TDE/16A exhibited a further depression of mating competency in the synchronous 34°C mating experiment (E3/E2 = 0.07). The *dna26-1* mutation in TDE/16A therefore caused a specific inhibition of mating under restrictive conditions. This inhibition was intermediate in its severity between that caused by the Class II *cdc35-1* and Class I *cdc36-16* START mutations.

INCORPORATION OF RADIOLABELLED AMINO ACIDS

The rate of incorporation of radiolabelled amino acids into cellular protein was chosen as a means of estimating the rate of cellular growth in the *dna26-1* mutant TDE/16A before and after a shift to the restrictive temperature. The amino acid incorporation rate of the *dna26-1* mutant was compared with that displayed by a wild type strain, a Class I START mutant and a Class II START mutant. The initial measurements carried out were for the incorporation of radioactive [U^{14}C]-arginine.

Incorporation of [U^{14}C]-arginine

The strain TDE/6B, which was derived from the same parents as TDE/16A, was used as the wild type control strain. A TDE/6B culture continued proliferation at a higher rate after the shift to 36°C than at 25°C (Figure 3.2). The doubling time (t_d) of the wild type culture was suprisingly low at 25°C compared with that of the other strains examined (t_d = 9.5hr, specific growth rate (μ) = 0.07.h^{-1}). The doubling time increased dramatically however after the shift to 36°C (t_d = 1.33hr, μ = 0.52.hr^{-1}). The budding index of the TDE/6B culture remained above 50% for up to 6hr after the shift to 36°C.



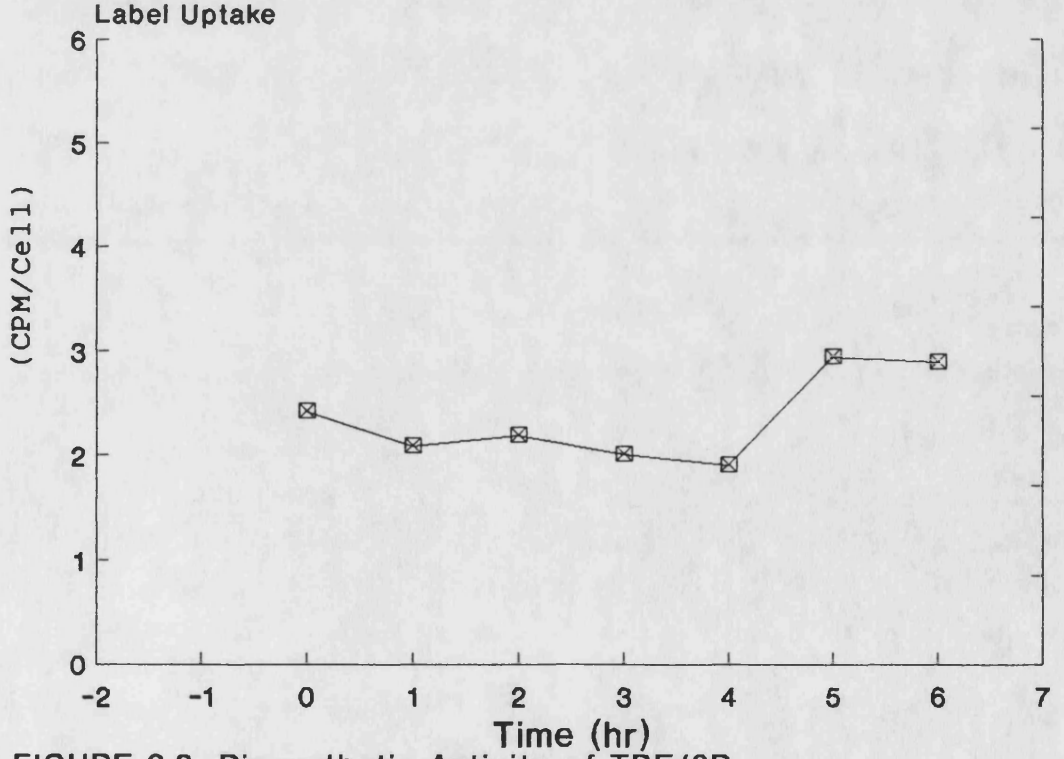


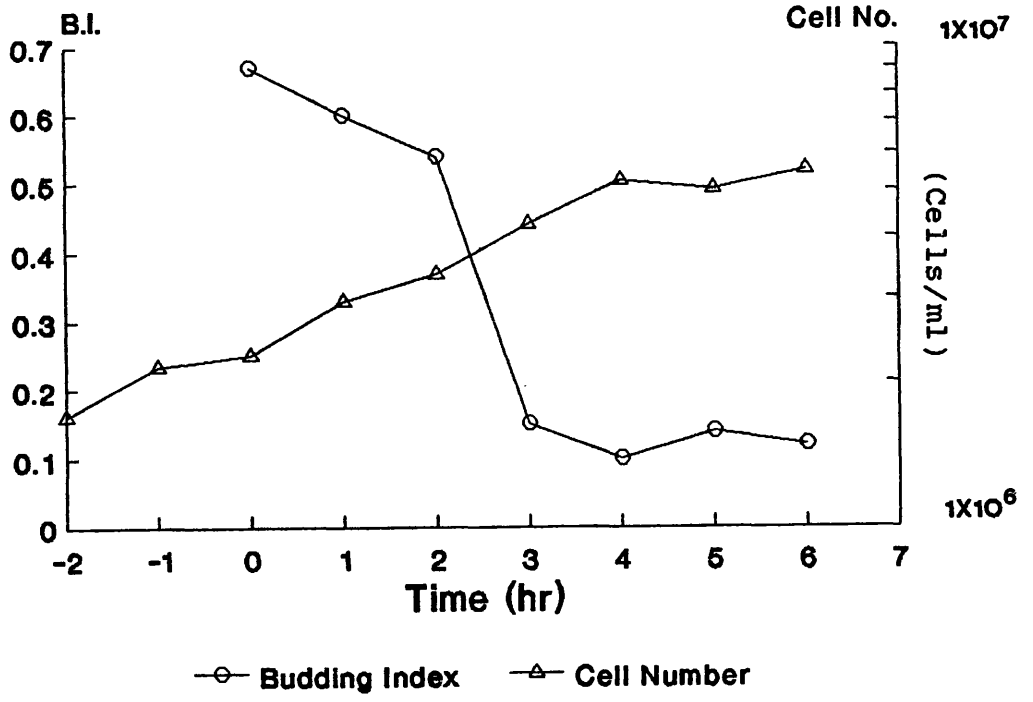
FIGURE 3.2 Biosynthetic Activity of TDE/6B

(Cells Shifted from 25°C to 36°C at Time t=0).

These results indicated that the culture was still growing at an exponential rate in the latter stages of the experiment. Supporting this was the observation that the rate of incorporation of radiolabelled arginine by the TDE/6B culture after 6hr of incubation at 36°C was similar to the rate of incorporation at the time of the temperature shift.

The Class I START mutant SR661-2(*cdc36-16*) was included as a positive control for continued protein synthesis during mutational START arrest. The *cdc36* mutant culture displayed a marked reduction in the rate of cell number increase when shifted from the permissive to the non-permissive temperature (Figure 3.3). Specifically, the doubling time for the culture at 25°C was 1.95hr (μ = 0.36.hr^{-1}) whilst at 36°C it was 4.95hr (μ = 0.14.hr^{-1}). The failure of the culture to cease proliferation completely revealed that the *cdc36-16* mutation was somewhat leaky in SR661-2. The mutant culture did however show a dramatic reduction in budding index after the shift to 36°C. Only 12% of the cells in the mutant culture displayed a bud after 6hr of incubation at 36°C indicating that most of the population was resident in the G1 interval of the cell cycle by this time.

At the time of the temperature shift the rate of label incorporation by the *cdc36* mutant culture was comparable to, though faster than that by the wild type strain TDE/6B. This higher initial incorporation rate by the mutant culture reflected its faster growth rate at 25°C compared with that of the TDE/6B culture. The incorporation of radiolabelled arginine by the mutant culture declined to 75% of its rate at 25°C by 6hr after the temperature shift suggesting that the *cdc36-16* mutation caused a gradual decline in cellular growth rate.



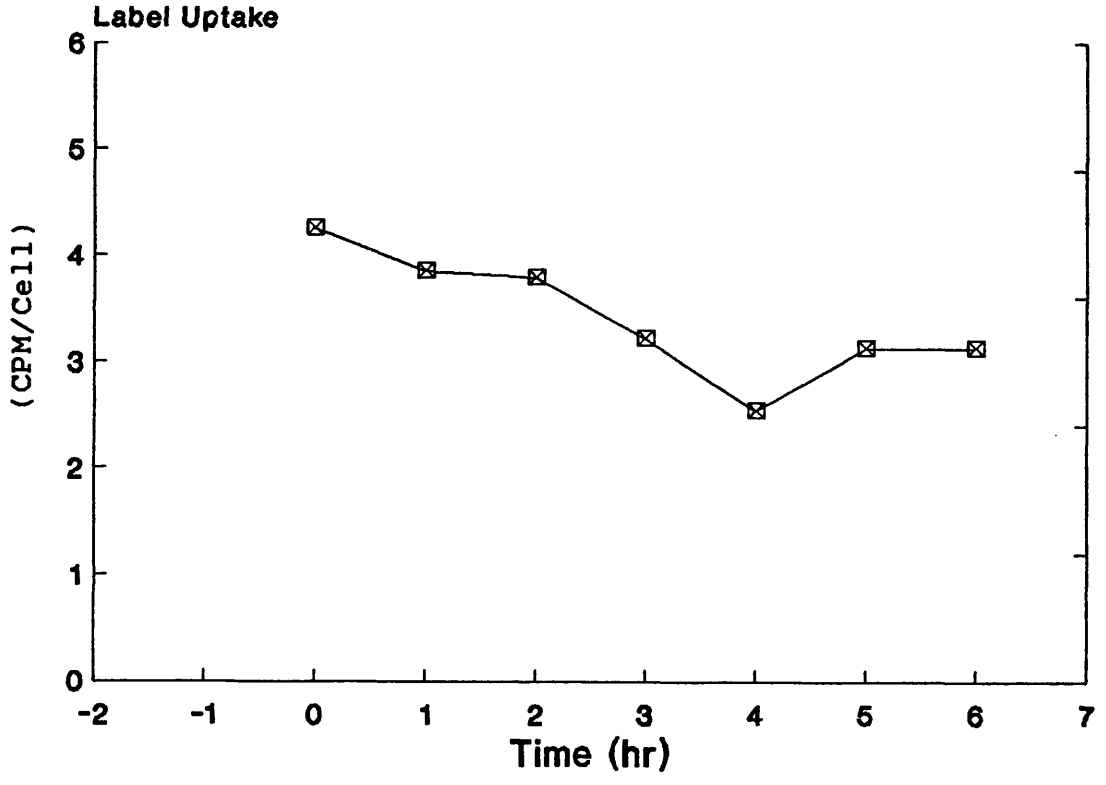


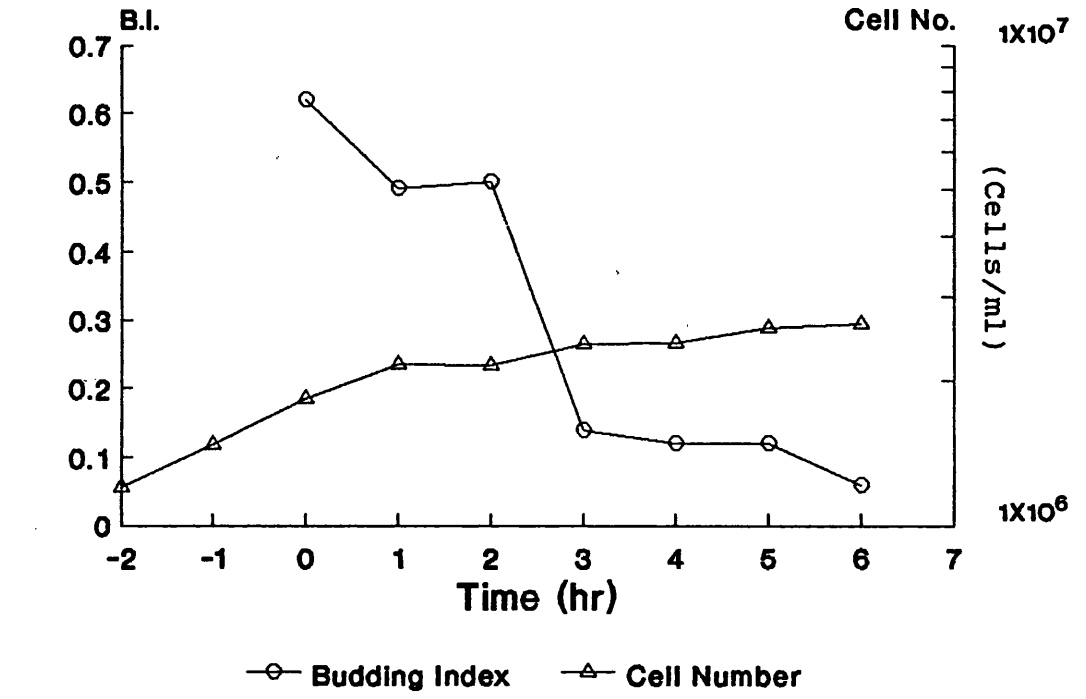
FIGURE 3.3 Biosynthetic Activity of SR661-2

(Cells Shifted from 25°C to 36°C at Time t=0).

The Class II START mutant BR35/7A(*cdc35-1*) was included as a negative control for growth during START arrest. The *cdc35* mutant culture displayed a rapid and dramatic reduction in the rate of cell number increase after the shift to the restrictive temperature (Figure 3.4). The doubling time for the mutant culture at 25°C was 2.85hr (μ = 0.24.hr^{-1}) whilst at 36°C it was 15.85hr (μ = 0.04.hr^{-1}). The arrest of proliferation by the *cdc35* mutant culture was accompanied by a rapid decline in the culture budding index from 0.62 at the time of the temperature shift to 0.06 by 6hr after the shift. These data indicated that the *cdc35-1* mutation was causing a tight START arrest in this strain.

As anticipated from its high specific growth rate at 25°C the *cdc35* mutant culture displayed a high rate of incorporaton of radiolabelled arginine at the permissive temperature (compared with the wild type strain and the *cdc36* mutant). In contrast to the *cdc36* mutant culture however, the rate of label incorporation by the *cdc35* mutant culture fell steadily over the 6hr of incubation at 36°C after the temperature shift. The final rate of incorporation by this strain after 6hr at 36°C was only slightly higher than background levels. Similar results have been obtained previously for a mutant containing the *cdc35-1* allele (Iida & Yahara, 1984a).

The *dna26-1* mutant TDE/16A displayed a reduction in the rate of cell number increase at 36°C that was comparable to that observed for the *cdc36* mutant (Figure 3.5). The decrease in the rate of proliferation occurred within one generation time after the shift to 36°C. The TDE/16A culture doubling time decreased from 2.4hr (μ = 0.29.hr^{-1}) at 25°C to 7.65hr (μ = 0.09.hr^{-1}) at 36°C. The failure of the mutant culture to cease proliferation was probably due to the leakiness of



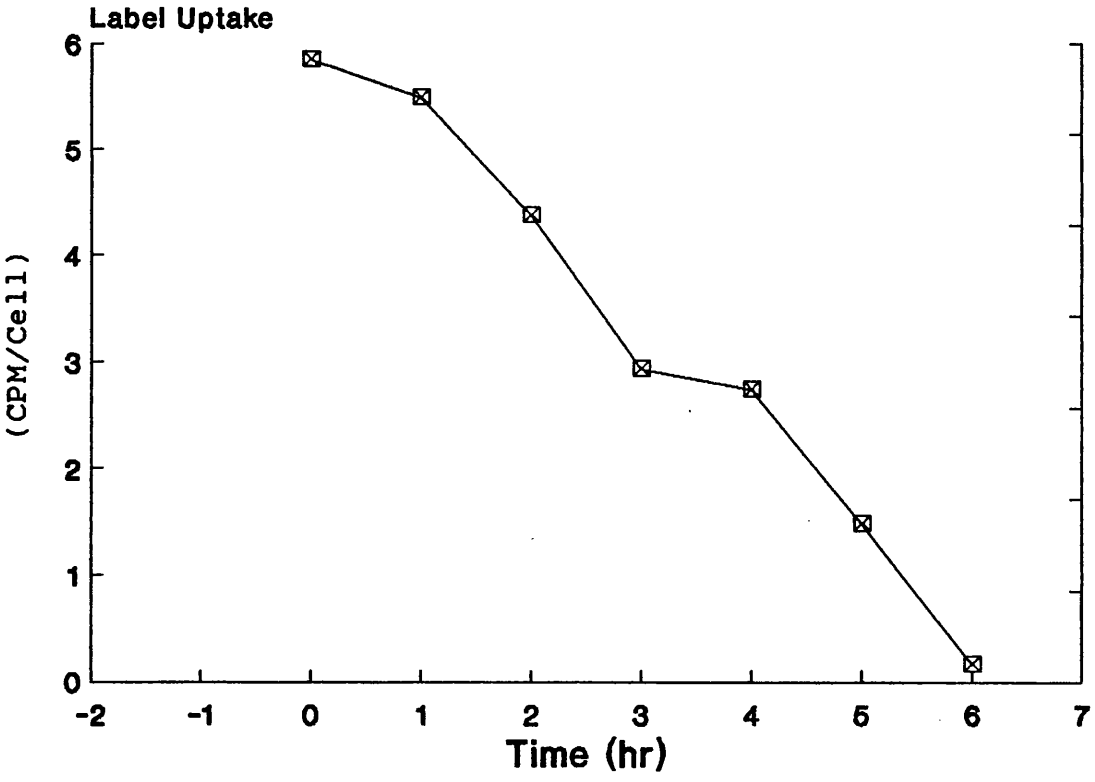
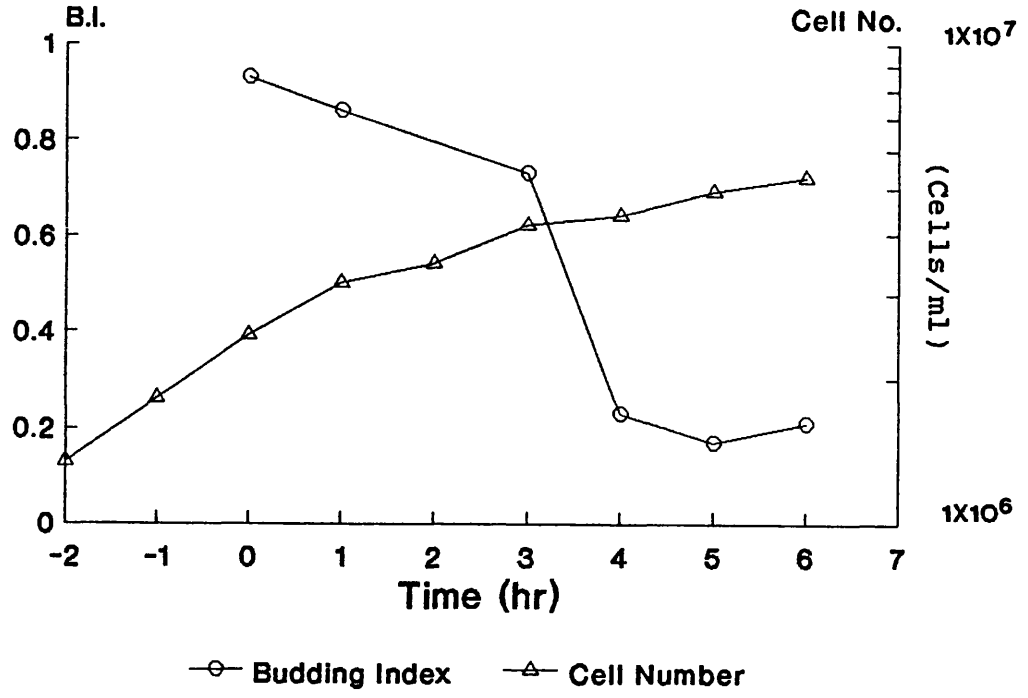


FIGURE 3.4 Biosynthetic Activity of BR214-4a

(Cells Shifted from 25°C to 36°C at Time t=0).



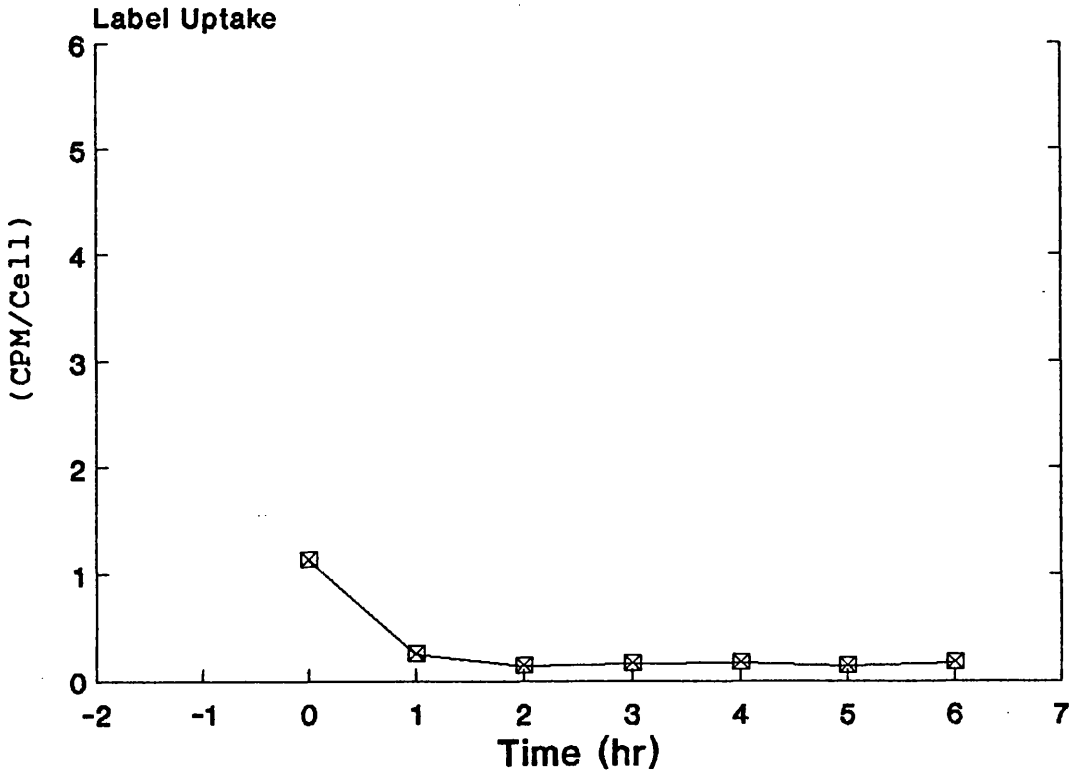


FIGURE 3.5 Biosynthetic Activity of TDE/16A

(Cells Shifted from 25°C to 36°C at Time t=0).

the *dna26-1* mutation in strain TDE/16A (see discussion, section 3.4). The budding index of the culture fell from 0.93 to 0.21 over the 6hr of incubation subsequent to the temperature shift. This also suggested that whilst the majority of the population was resident in G1 at the end of the experiment, nevertheless some cells may have reinitiated a new cell cycle at the restrictive temperature.

In contrast to the incomplete arrest of proliferation at the restrictive temperature the *dna26-1* mutant displayed a dramatic reduction in the rate of incorporation of radiolabelled arginine after the shift to 36°C. The fall in the incorporaton rate was also very rapid. The second 15min sampling period, carried out 1hr after the temperature shift, measured an incorporation rate that was only 22% of the rate estimated by the first 15min sample taken at the time of the shift. By the third sample, taken 2hr after the temperature shift, the rate of incorporation had fallen to and levelled off at 12.2% of the incorporation rate measured in the sample taken at the time of the temperature shift. Due to the rapid response of the *dna26-1* mutant to the temperature shift and the 15min sampling period employed in these assays, the original rate of label incorporaton by the *dna26-1* mutant at 25°C could not be accurately judged from these data.

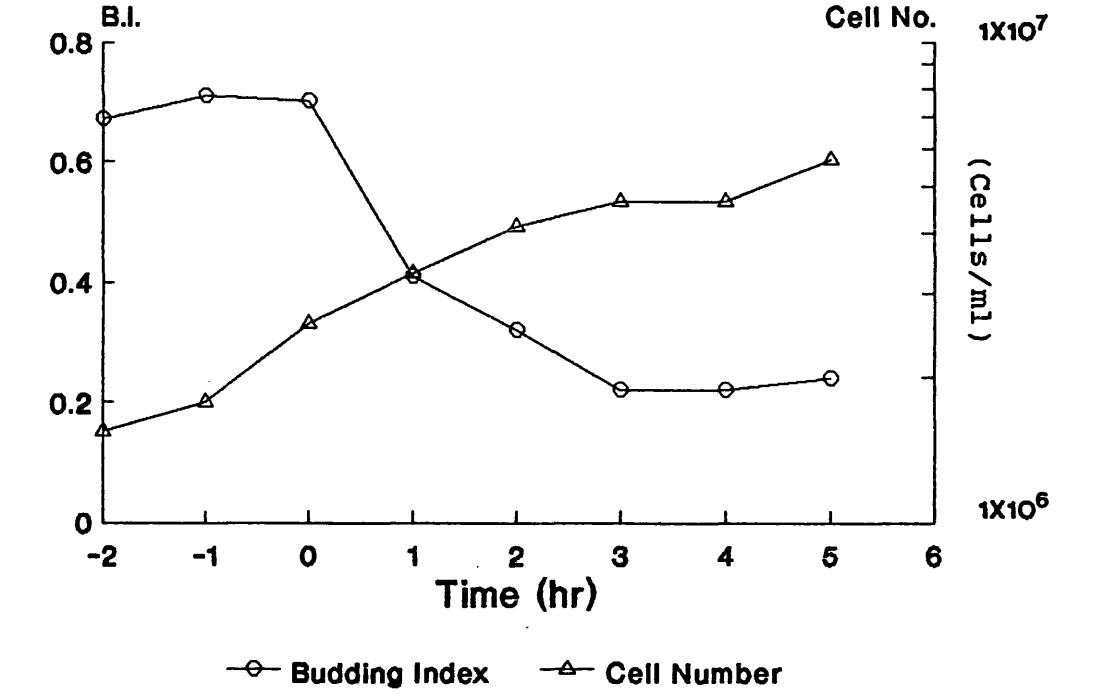
Because the rate of radiolabelled arginine incorporaton by the *dna26-1* mutant even under permissive conditions appeared to be markedly slower than that exhibited by the control START mutants, a separate experiment was carried out. In this second experiment the volume of radiolabelled arginine added to the TDE/16A sample tube was increased from 10µl (0.5µCi) to 40µl (2.0µCi) whilst all other conditions were identical to those in the first experiment.

The effect of a temperature shift from 25°C to 36°C in the second experiment had a similar effect on proliferation of the TDE/16A culture as seen in the first experiment (Figure 3.6). The budding index of the *dna26-1* mutant culture fell from 0.67 at the time of the shift to 0.24 after 4hr at 36°C. The culture doubling time decreased from 2.55hr (μ = 2.7.hr^{-1}) at 25°C to 7.45hr (μ = 0.09.hr^{-1}) at 36°C. The 15min label incorporation measurement taken immediately after the shift to 36°C displayed a 33% reduction in the rate of incorporation at 25°C. By 1hr after the shift to 36°C the rate of label incorporation had fallen to approximately 10% of the rate measured at 25°C. The final rate of incorporation of radiolabelled arginine was 13-fold higher than background levels of incorporation (data not shown).

Incorporation Rate of a [U^{14}C]-labelled Protein Hydrolysate

Vacuolar stores of arginine are thought to be released in *S. cerevisiae* during starvation conditions (Sumrada & Cooper, 1978). It was therefore considered possible that the decrease in arginine incorporation observed in the initial labelling experiments might have been due to dilution of the radioactive arginine with unlabelled arginine from intracellular stores. It was also considered possible that the results obtained in the first assay might be specific to arginine incorporation for other reasons such as a possible specific effect of the *dna26-1* mutation on the arginine permease. A second set of experiments was therefore carried out in which a [U^{14}C]-labelled protein hydrolysate was employed instead of [U^{14}C]-arginine.

The wild type strain TDE/6B continued proliferation after a shift from 24°C to 36.5°C, although a decelaration in the exponential



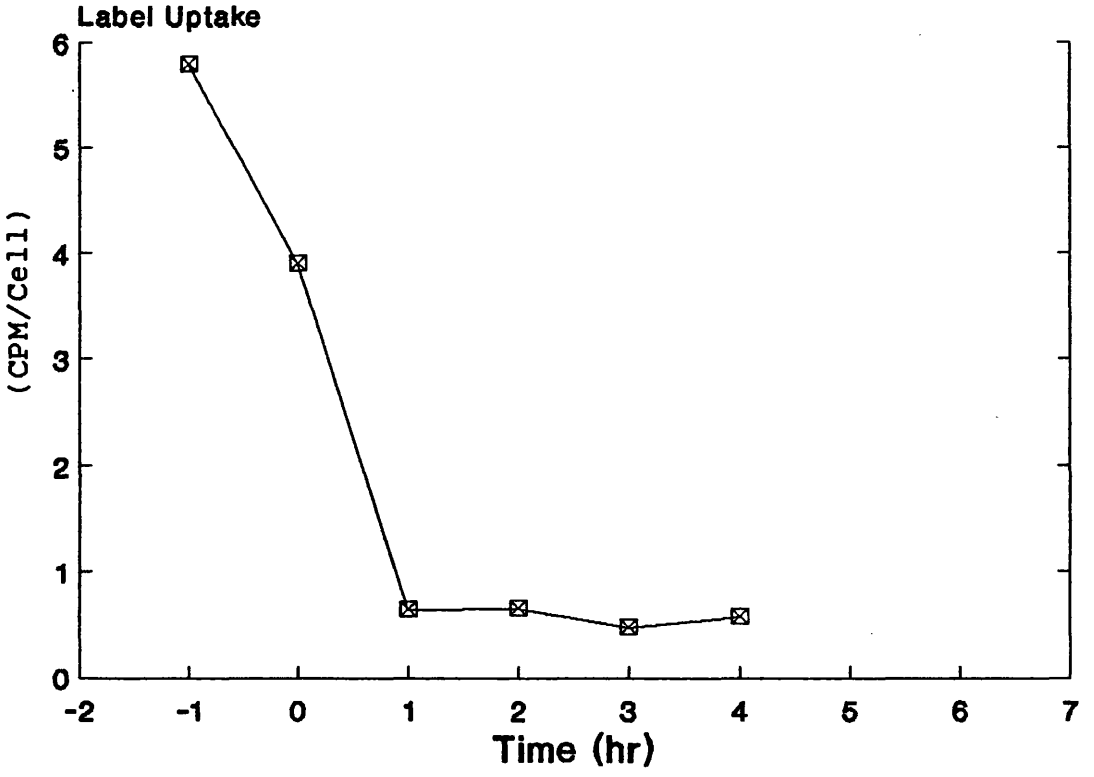


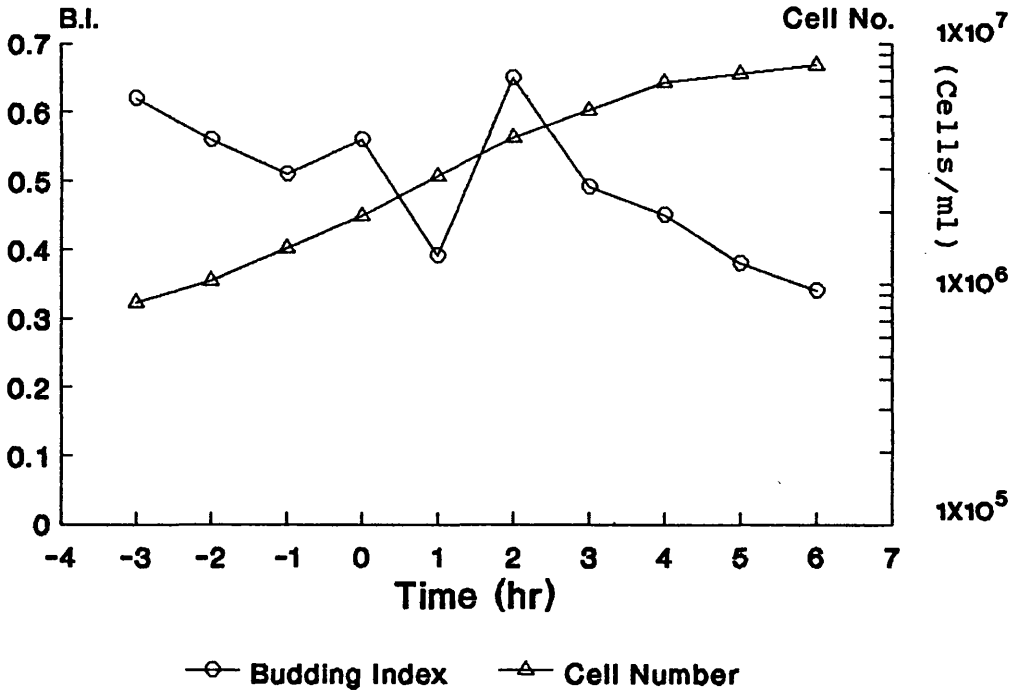
FIGURE 3.6 Biosynthetic Activity of TDE/16A

Conditions identical to those in Figure 3.5 except for a 4-fold increase in the volume of [U^{14}C]-arginine added to sampling tubes. (Cells shifted from 25°C to 36°C at Time t=0).

growth of the culture was observed after 5hr at the higher temperature (Figure 3.7). The decline in the growth rate began to occur when the culture density was still quite low ($7X10^6$/ml) indicating that the growth of the strain was probably adversely affected at the higher temperature. It is also possible however that the culture became limited for one of the growth supplements. The budding index also declined gradually from approximately 60% at 24°C to 40% by 5hr after the shift to 36.5°C. The doubling time of the culture remained approximately constant in the early part of the experiment at 2.15hr ($\mu = 0.32.hr^{-1}$).

A transient decline in the rate of label incorporation was observed approximately 1hr after the temperature shift reflecting a transient decrease in the budding index of the culture at this time. A transient fall in budding index in cultures experiencing a shift-up of temperature has been reported previously (Johnston & Singer, 1980). The rate of radiolabelled amino acid incorporaton returned to its original rate by the second hour after the temperature shift. After 4hr at 36.5°C the rate of label incorporaton began to decline again, reflecting a fall in the culture budding index and rate of cell number increase.

The incorporation of the radiolabelled protein hydrolysate was also monitored in a *cdc36* and a *cdc35* START mutant. The mutants showed a qualitatively similar capability for the incorporaton of the protein hydrolysate at the restrictive temperature as they did for the radiolabelled arginine in the first experiment. The *cdc36* mutant maintained a high level of label incorporation at 36.5°C whilst incorporation by the *cdc35* mutant declined steadily after the temperature shift (data not shown).



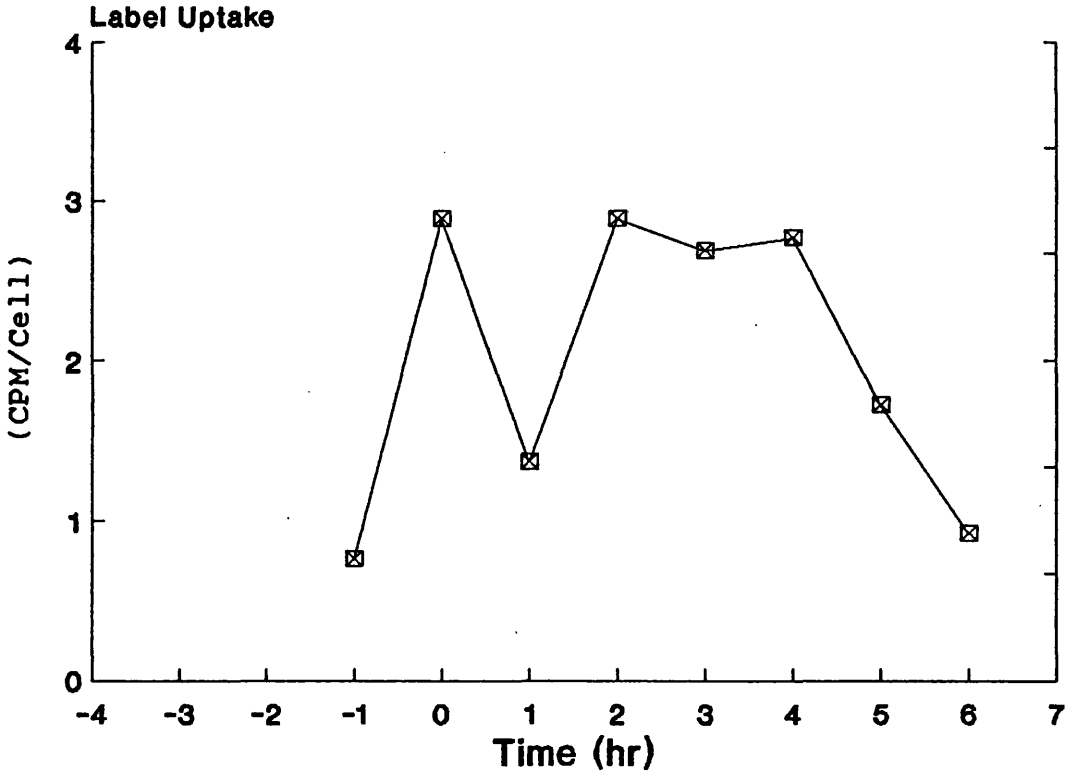


FIGURE 3.7 Biosynthetic Activity of TDE/6B

(Cells Shifted from 24°C to 36.5°C at Time t=0).

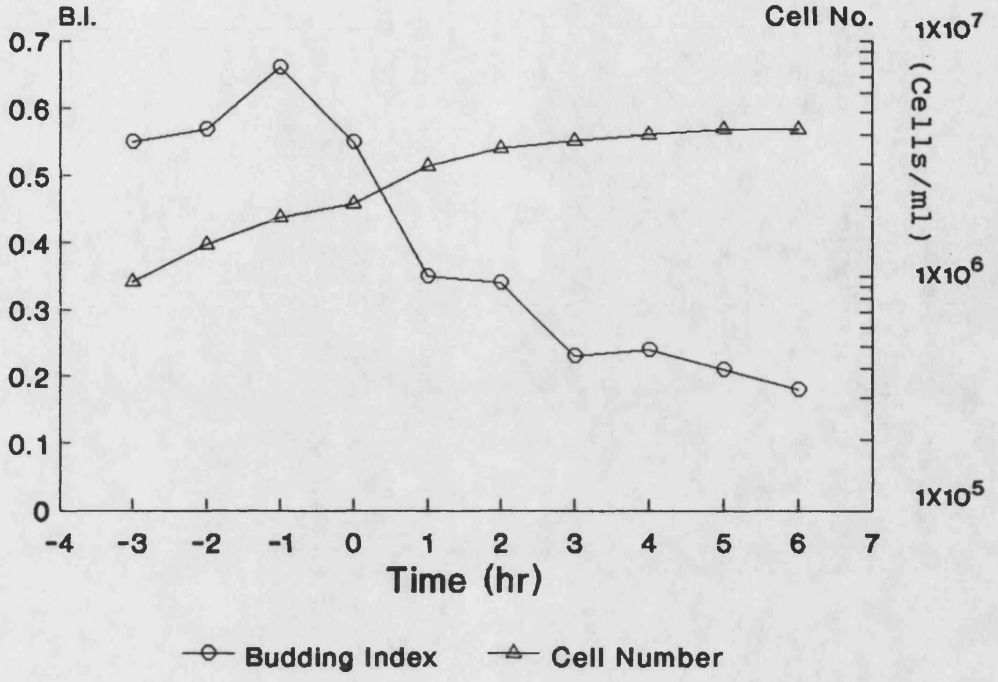
The doubling time of the *dna26-1* mutant culture again declined from 2.5hr (μ = 0.28.hr^{-1}) at 24°C to 12.58hr (μ = 0.06.hr^{-1}) after the temperature shift to 36.5°C (Figure 3.8). The culture budding index fell from 0.55 at the time of the shift, to 0.18 after 5hr at 36.5°C. During the first 10min sampling period after the shift to 36.5°C the rate of incorporation of radiolabelled amino acids by the *dna26-1* mutant fell to 84.8% of the rate of incorporation at 24°C. By 2hr after the shift to 36.5°C the rate of incorporation had fallen to 18% of the rate at 24°C. Incorporation of the labelled hydrolysate continued at this level until the end of the experiment 5hr after the temperature shift.

In summary a similar result was obtained from both the experiment using [^{14}C]-arginine and that using the [^{14}C]-labelled protein hydrolysate. In both cases the rate of radiolabelled amino acid incorporation by the *dna26-1* mutant TDE/16A decreased to less than 20% of the initial rate within 1-2hr of a temperature shift from the permissive to the non-permissive temperature.

THERMOTOLERANCE DURING START ARREST

The resistance of the *dna26-1* mutant TDE/16A to heat killing during cell cycle arrest at 36.5°C was assessed by exposing the mutant cells to a 52°C heat shock for 10min. The response of TDE/16A to heat shock was once again compared to that of a wild type strain, a Class I START mutant and a Class II START mutant exposed to identical conditions. The results are presented in Figure 3.9 and reiterated in the text.

The strain MD40/4C had been used as the wild type parent during the



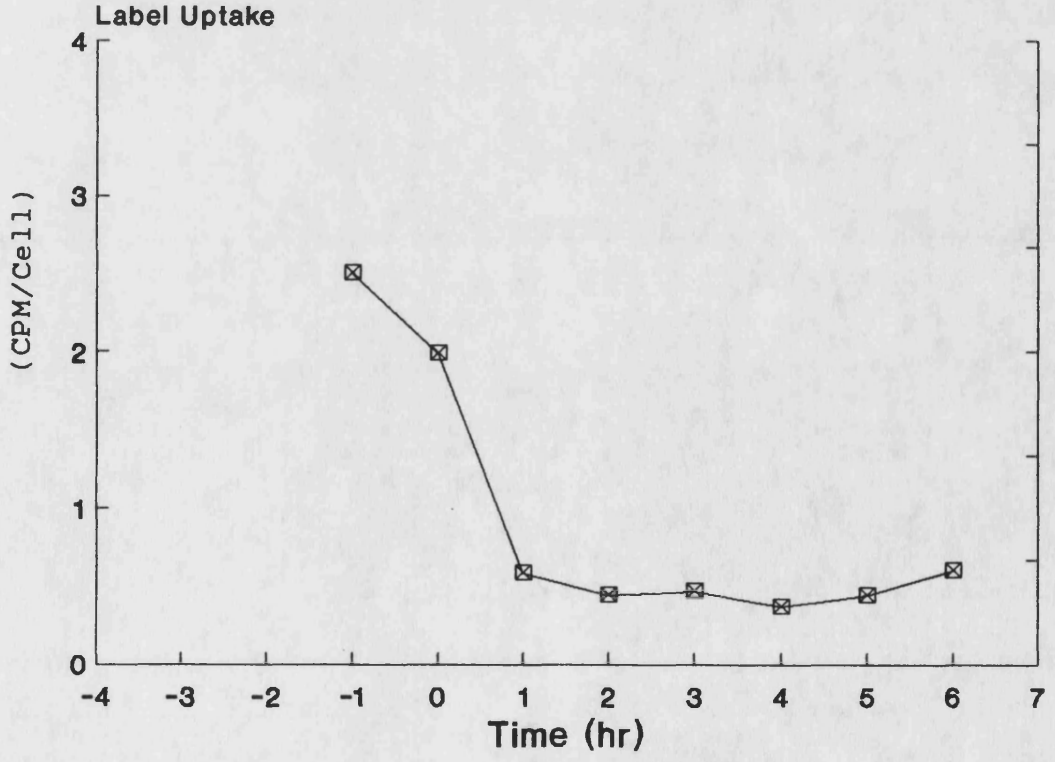


FIGURE 3.8 Biosynthetic Activity of TDE /16A

(Cells Shifted from 24°C to 36.5°C at Time t=0).

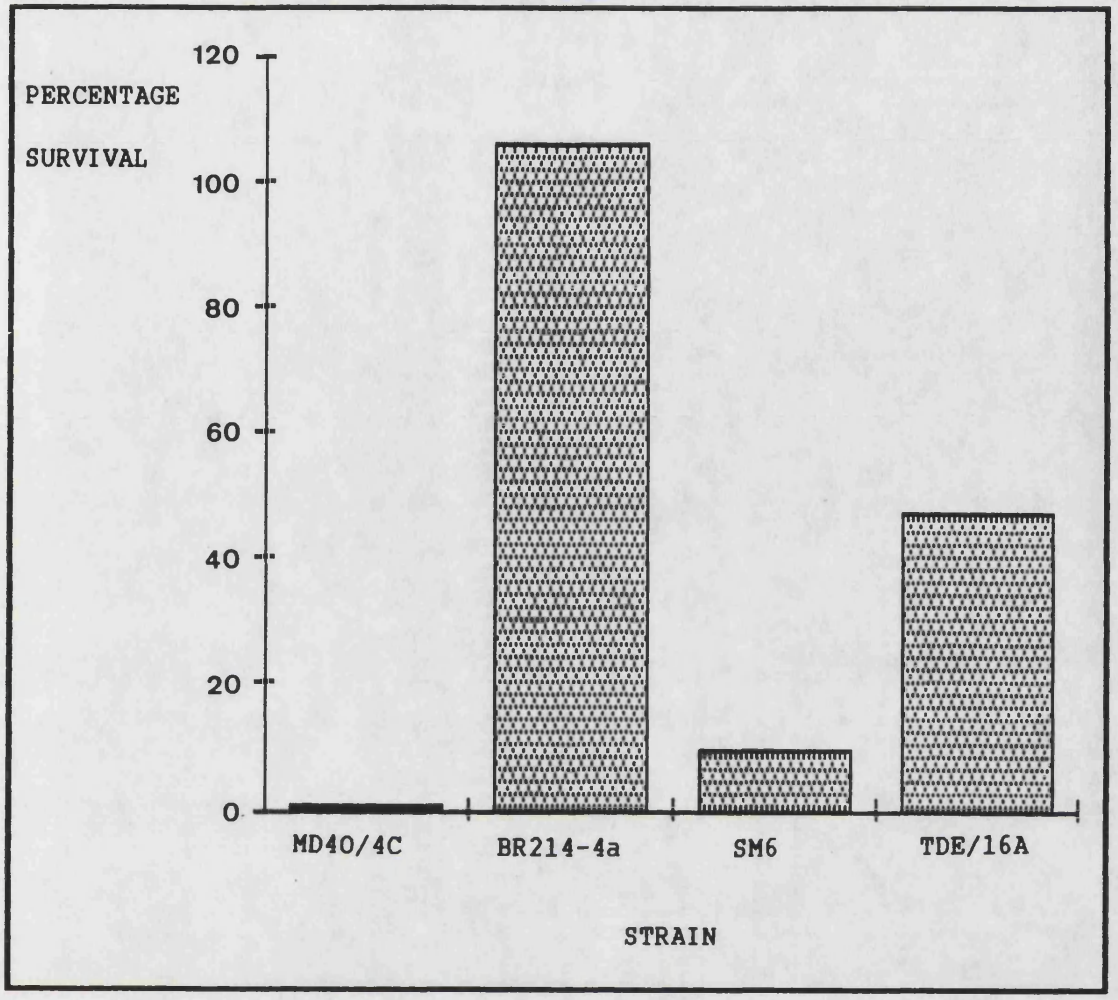


FIGURE 3.9

Heat Shock Resistance During START Arrest

The figure shows the percentage survival of various strains after an exposure to heat shock sustained during mutational START arrest. The percentage survival for each strain was calculated from the number of viable cells before and after exposure to 52°C for 10min. MD40/4C was included as a wild type control and was therefore not arrested at START at the time of the heat shock. BR214-4a contains the *cdc35-1* mutation and was included as a control Class II START mutant. SM6 contains the *cdc28-6* mutation and was included as a control Class I START mutant. TDE/16A contains the *dna26-1* mutation.

construction of TDE/16A. MD40/4C therefore provided a suitable control for analysing the response of a wild type strain to heat shock. When an MD40/4C culture had reached the exponential phase of growth it was shifted from a growth temperature of 25°C to 36.5°C. After 5hr at 36.5°C the budding index of the MD40/4C culture was 0.63 suggestive of ongoing proliferation. When exposed to a heat shock of 52°C for 10min the culture showed a survival rate of 0.28% revealing a high susceptibility of the wild type culture to heat killing at 52°C despite the preincubation at 36.5°C.

The Class II START mutant BR214-4a(*cdc35-1*) was included as a positive control for the acquisition of heat shock resistance during START arrest. When an exponentially growing culture of BR214-4a cells was shifted from 25°C to 36.5°C the culture budding index fell from an initial value of 0.49 to a final value of 0.09 after 5 hours (data not shown). This result suggested that the *cdc35-1* mutation was causing a tight START arrest. The START arrested BR214-4a culture displayed a survival rate of 105.51% after a heat shock at 52°C for 10min. The survival rate of greater than 100% obtained was probably due to practical inaccuracies during the dilution and spread-plating of the heat shocked cells prior to viability estimates. A comparable level of heat shock resistance has been reported previously for BR214-4a (Plesset *et al.*, 1987).

The Class I START mutant SM6(*cdc28-6*) was included as a negative control for the acquisition of heat shock resistance during START arrest. After the shift of an exponentially growing culture of SM6 cells from 25°C to 36.5°C the budding index of the culture fell from 0.6 to 0.07 within 5hr (data not shown) suggesting that the *cdc28-6* mutation was causing a tight START arrest in this strain. The SM6

culture displayed a survival rate of 9.19% after exposure to 52°C for 10min. This survival rate was 30-fold higher than that shown by the wild type strain MD40/4C suggesting that the START arrest conferred by the *cdc28-6* mutation was causing a limited resistance to heat shock. SM6 is however genetically unrelated to MD40/4C. Uncertainty about the influence of genetic background on the acquisition of thermotolerance therefore precluded an unequivocal association of the *cdc28-6*-induced START arrest with induction of heat shock resistance. In light of the results obtained with the *cdc35* mutant it was concluded that the *cdc28-6* mutant was displaying a low level of resistance to heat killing during START arrest. This type of response is characteristic of a Class I START mutant (Plesset *et al.*, 1987).

After temperature shift of an exponentially growing culture of the *dna26-1* mutant TDE/16A from 25°C to 36.5°C the culture budding index fell from an intial value of 0.67 to a value of 0.24 after 5hr. This final value for culture budding index suggested that whilst the majority of the cells in the TDE/16A culture were resident in the G1 phase of the cell cycle, cellular proliferation had not ceased completely at the restrictive temperature. After a heat shock at 52°C for 10min the TDE/16A culture displayed a survival rate of 47.36%. This degree of thermotolerance was intermediate to that shown by the Class I and Class II START mutant. This result may have been an underestimation of the heat shock resistance of the *dna26-1* strain during START arrest. This was due to the fact that in this particular experiment the budding index of the culture did not fall to as low as its usual value of less than 0.2 after a period of 5hr at 36.5°C.

3.4 DISCUSSION

The Kinetics of Cell Cycle Arrest in *dna26-1* Mutant Cells

During a temperature shift from 24°C to 37°C the *dna26-1* mutant TDE/16A displayed a rapid decrease in the rate of cellular proliferation that was accompanied by a fall in the culture budding index. This response is similar to that displayed by *cdc* mutants defective in the execution of START which arrest in G1 at the restrictive temperature.

The mutant strain TDE/16A was derived from the *dna26-1* mutant JL448 by repeated backcrossing to a wild type strain and selection for the temperature sensitive G1-arrest phenotype (section 2.3). A comparison of the kinetics of cell cycle arrest in the two mutant strains TDE/16A and JL448 revealed that the conditional cell cycle arrest in the primary mutant JL448 was tighter than in the purified mutant TDE/16A. The percentage of budded cells in JL448 cultures typically falls to about 10% after 3-4hr at the restrictive temperature (see Tables 2.6 and 2.8, section 2.3). In comparison the percentage of budded cells in TDE/16A culture falls more gradually to approximately 20% after 5hr at 37°C (Figure 3.1A). During its original identification, JL448 was exposed to mutagenic treatment with ethyl methane sulphonate (Dumas *et al.*, 1982). The tighter conditional arrest observed in this strain compared with TDE/16A may therefore be due to additional mutations that exacerbate the temperature sensitive phenotype caused by the *dna26-1* mutation. These secondary mutations may have been diluted out by the backcrossing procedure leading to the less severe temperature sensitive lesion observed in TDE/16A. Alternatively the MD40/4C

genetic background may partially suppress the effect of the *dna26-1* mutation in TDE/16A. Comparison of the *dna26-1* mutant phenotype in a range of genetic backgrounds may be required to resolve the differences observed between JL448 and TDE/16A.

The *dna26-1* Mutation Effects Incorporation of Amino Acids Into Cellular Protein

The *dna26-1* mutant TDE/16A displayed a rapid and severe decrease in the rate of uptake of radiolabelled amino acids after a shift to restrictive conditions. The cell cycle arrest phenotype of this mutant is therefore probably the consequence of a temperature sensitive defect in protein synthesis caused by the *dna26-1* mutation. The experiments using ^{14}C-arginine did not however rule out the possibility that the reduction in the rate of label incorporation at 36°C was due to a defect in amino acid transport into the cell or changes in the intracellular pool of arginine.

During the amino acid incorporation experiments yeast cultures were grown on SD medium containing 5g/l of $(NH_4)_2SO_4$. At this ammonium concentration the general amino acid permease, which facilitates the uptake of all basic and neutral amino acids into *S. cerevisiae* cells, is repressed (Woodward & Cirillo, 1977). Uptake of arginine during the labelling experiments was therefore via the arginine permease encoded by the *CAN1* gene. Because the experiments using a ^{14}C-labelled protein hydrolysate gave comparable results to those employing ^{14}C-arginine, it is unlikely that the *dna26-1* mutation has a specific effect on the arginine permease.

The experiments using the radiolabelled protein hydrolysate cannot rule out the argument for dilution of the radiolabelled amino acids

by the release of intracellular amino acid stores. It is possible that the *dna26-1* mutation causes a starvation response in the cell which would explain the conditional G1 arrest phenotype. During such a response the cell would begin rapid degradation of existing proteins to release amino acids for new protein synthesis (Johnston *et al.*, 1977b). This would lead to dilution of radiolabelled amino acids by non-labelled amino acids resulting in an overestimation of the protein synthesis defect by this assay method. Resolving these arguments will require analysis of the effect of the *dna26-1* mutation on the intracellular amino acid pool size in mutant cells.

Protein synthesis did not cease completely at 36°C in the *dna26-1* mutant TDE/16A. The continuing low rate of amino acid incorporation several hours after the shift to restrictive conditions may explain the *cdc* phenotype of this strain. Only low levels of protein synthesis are thought to be required for a cell to complete the cell cycle after traversing START (Johnston *et al.*, 1977a). Growth to a critical cell size is however thought to be required for execution of START (Hartwell & Unger, 1977). A possible explanation for the G1 arrest phenotype of the *dna26-1* mutant at 37°C is therefore that the cells are able to complete the current cell cycle at the restrictive temperature but are unable to attain the critical cell size for START because of insufficient mass accumulation.

The gradual fall in the budding index of TDE/16A cultures at 37°C is probably also the result of the severe but incomplete inhibition of protein synthesis at this temperature. Some degree of ongoing protein synthesis is required for progression through the cell cycle even after START (Johnston *et al.*, 1977b; Unger & Hartwell, 1976). The low rate of protein synthesis achieved by TDE/16A as early as 1hr

after a shift to restrictive conditions may therefore permit only a slow progression through the cell cycle. By this argument, all the cells in the mutant population that have completed START at the time of a temperature shift complete their current cell cycle at a slow rate before arresting in G1. This slow cell cycle progression is manifested in a gradual decline in the culture budding index for TDE/16A over a period of 5hr.

The TDE/16A mutant population may not completely arrest at START however. Even after 5hr at 37°C, a slow increase in cell number was still observed in the mutant culture and 20% of the cells displayed a bud. The mutant population may therefore be undergoing a protracted G1 accumulation rather than a complete START arrest at the restrictive temperature. G1 accumulation occurs as a consequence of the differential sensitivity of the START regulatory step to protein synthesis compared with other stages in the cell cycle (Shilo *et al.*, 1978b) combined with the size requirement for START. The cells in the TDE/16A culture may therefore be slowly accumulating mass during their long residence in G1. Finally when the cells attain the minimum size for cell cycle progression they traverse START and begin a new cell cycle. By this means the budding index for the TDE/16A culture remains at a value of approximately 0.2.

The characteristics of the response of TDE/16A to the restrictive temperature preclude its rigid classification as a START mutant. The *dna26-1* allele behaves differently however in the two genetic backgrounds examined to date. The primary mutant JL448 displays a tighter START arrest at 37°C than the slow G1 accumulation observed in cultures of TDE/16A. Introduction of the *dna26-1* mutation into an alternative genetic background may therefore be required to

properly establish its affect on the regulatory mechanisms operating at START.

Additionally, although experiments on the original *dna26-1* mutant indicated that it was arresting at a stage in the cell cycle prior to the initiation of DNA synthesis (Dumas *et al.*, 1982), a strict classification of *dna26-1* as a START mutation will require confirmation of its prevention of DNA synthesis in a purified *dna26-1* mutant. Such confirmation may be provided by analysing the DNA content of a *dna26-1* mutant before and after a shift to restrictive conditions by propidium iodide staining and flow cytometry. A mutant displaying a tighter conditional arrest than TDE/16A or an alternative mutant allele of *DNA26* will be required to provide conclusive results by such means.

Finally, the very rapid inhibition of protein synthesis observed in TDE/16A after a shift to restrictive conditions suggests that the mutant *dna26-1* gene product is thermolabile. The continued low rate of protein synthesis observed in TDE/16A at the restrictive temperature suggests that either the mutant gene product retains a low level of activity at the elevated temperature or that it is dispensible for a low rate of protein synthesis.

The *dna26-1* Mutation Affects Mating Ability

Examination of the conjugational competence of the mutant TDE/16A during cell cycle arrest revealed an inhibition of mating specifically caused by the *dna26-1* mutation. This result provides evidence against the involvement of the *DNA26* gene in START arrest as part of the mating pheromone signal transduction pathway. The result is consistent with the observation that the *dna26-1* mutant fails to

form shmoos at the restrictive temperature and that a diploid homozygous for *dna26-1* displays a G1 arrest at the restrictive temperature (section 2.3). The decrease in conjugational competence in the *dna26-1* mutant TDE/16A at 34°C is probably a result of its reduced capacity for protein synthesis under restrictive conditions. Several processes specific to the mating response are dependent upon continued protein synthesis. These include the synthesis of cell surface agglutinins (Fehrenbacher *et al.*, 1978; Betz *et al.*, 1978), the synthesis of proteins that promote cell fusion (McCaffrey *et al.*, 1987) the synthesis of peptide mating pheromone (Scherer *et al.*, 1974) and the turnover of pheromone receptors (Jenness & Spatrick, 1986).

The reduction in mating competency caused by the *dna26-1* mutation at 34°C was intermediate in severity to that caused by a Class I and Class II START mutation. The *dna26-1* mutation may be somewhat leaky at the restrictive temperature of 34°C used in the mating assays. The inhibition of mating by the *dna26-1* mutation may therefore have been underestimated by these experiments. The high level of mating competency reported for JL448 at 34°C (Green, 1986) is difficult to reconcile with the tighter START arrest seen in this strain compared with that in TDE/16A. If, as suspected, the START arrest caused by the *dna26-1* mutation is due to a protein synthesis defect, the tighter arrest in JL448 would be expected to cause a greater inhibition of mating because of a more severe inhibition of protein synthesis. Further analysis of the two *dna26-1* mutant strains will be required to explain their different behaviour.

The *dna26-1* Mutation Causes Limited Acquisition of Thermotolerance

Under restrictive conditions the *dna26-1* mutant TDE/16A displayed a resistance to heat killing that was intermediate to that exhibited by a Class I and a Class II START mutant. The Class II *cdc35-1* mutant employed in the heat shock experiments possesses a thermolabile adenylyl cyclase enzyme (Boutelet *et al.*, 1985). At the restrictive temperature a *cdc35* mutant arrests at START in a stationary phase-like state due to defective cAMP-mediated nutrient signalling (Iida & Yahara, 1984a; Gibbs & Marshall, 1989). In this state it acquires the thermotolerance characteristic that is typical of stationary phase cells. The *dna26-1* mutation did not appear to induce a stationary phase-like thermotolerance in TDE/16A to the same degree that the *cdc35* mutation did in the mutant BR214-4a. However explanation of the phenotype of the *dna26-1* mutant is again complicated by the apparent leakiness of the mutation in TDE/16A.

The *dna26-1* mutation in TDE/16A may cause a leaky starvation signal at 36.5°C. Although protein synthesis was severely inhibited in this strain, nevertheless proliferation did not completely arrest under restrictive conditions. The intermediate level of heat shock resistance acquired by TDE/16A during the thermotolerance assay may therefore have been due to the fact that only a sub-population of the mutant culture entered stationary phase from G1 during the preincubation at 36.5°C. It has been suggested that even in an exponentially growing culture of wild type cells a fraction of the population may enter G0 in a stochastic fashion (Plesset *et al.*, 1987). The slow rate of progression through G1 by cells of TDE/16A whilst experiencing an inhibition of protein synthesis may have increased the probability of cells entering G0 and acquiring

thermotolerance.

The stress imposed on cells of strain TDE/16A by the protein synthesis defect at the restrictive temperature can provide an alternative explanation for the thermotolerance acquired by the *dna26-1* mutant culture. Physiological characteristics such as resistance to cell wall-degrading enzymes, accumulation of storage carbohydrates and thermotolerance are usually associated with stationary phase. However it has been suggested that these characteristics are merely a result of the physiological stress experienced by the cell during, for example, starvation rather than characteristics of stationary phase itself (Drebot *et al.*, 1990). The *dna26-1* mutation may therefore be causing physiological stress at the restrictive temperature but not a starvation response, resulting in the limited acquisition of thermotolerance observed.

Other Mutations that Affect Both START and Protein Synthesis

Mutations affecting the biosynthesis of amino acids, the charging of transfer RNA (tRNA) and the translation of messenger RNA into protein have been found that also cause conditional START arrest. The *gcd1-1* and *gcd12* mutations cause derepression of the *GCN4* gene involved in the general control of amino acid synthesis. Both of these mutations can also cause G1 arrest at 36°C. The temperature sensitive *gcd1-1* mutation has been shown to cause a rapid decrease in protein synthesis upon shift to 36°C (Hill & Struhl, 1988). The *GCD1* gene is allelic to the *TRA3* gene (Wolfner *et al.*, 1975) and has been cloned and sequenced. Although the DNA sequence of the *GCD1* gene shows no homology to any other cloned gene it has been suggested that the *GCD1* gene product may be an integral component of the translational

machinery (Hill & Struhl, 1988). The *gcd12* and *gcd2-1* mutations are alleles of the same gene (Paddon & Hinnebusch, 1989). Whereas the *gcd12* mutation can cause START arrest at 36°C in the presence of a *gcn3* mutation (Harashima *et al.*, 1987) the *gcd2-1* mutation merely causes a slow growth phenotype.

The *ils1-1* (Hartwell & McLaughlin, 1968) and *mes1-1* (Unger & Hartwell, 1976) mutations encode a thermolabile isoleucyl- and methionyl-tRNA synthetase respectively. At the restrictive temperature the *ils1-1* mutation causes a complete cessation of cellular proliferation at START (Niederberger *et al.*, 1983). In contrast, the *mes1-1* mutation causes mutant cells to accumulate in G1 without a complete cessation of proliferation (Johnston & Singer, 1990). The *alg1-1* mutation similarly causes G1 accumulation of mutant cells at the restrictive temperature (Klebl *et al.*, 1984). Strains containing a mutation in the *ALG1* gene are defective in the N-glycosylation of proteins.

Mutations in two *CDC* genes, *cdc33-1* and *cdc63-1*, that are thought to be involved in translational initiation also cause conditional START arrest. *CDC63* has been found to be allelic to the *PRT1* gene (Hanic-Joyce, 1985). The *prt1-1* mutation has been shown to cause a defect in the formation of the 40S preinitiation complex (Feinberg *et al.*, 1982). Several alleles of the *PRT1* gene have been isolated (Hartwell & McLaughlin, 1969; Thonart *et al.*, 1976; Bedard *et al.*, 1981) and under appropriate restrictive conditions each allele has been shown to be capable of causing conditional START arrest (Hanic-Joyce *et al.*, 1987a). The *PRT1* gene has been cloned and sequenced and shows no homology to any other known protein (Hanic-Joyce *et al.*, 1987b). The *cdc33-1* mutation was identified as causing a conditional G1

arrest phenotype in mutant cells (Pringle & Hartwell, 1981). The *CDC33* gene has been cloned and sequenced and revealed to be the *S. cerevisiae* initiation factor eIF4-E cap-binding protein (Brenner *et al.*, 1988). The latter authors have suggested that a cell cycle regulatory role for the eIF4-E protein may be mediated via the differential translation of mRNA's in response to the nutrient status of the cell.

The Role of Protein Synthesis in the Regulation of the Cell Cycle

Mutations in the *PRT1* gene can cause arrest of proliferation at START despite the maintenance of a level of protein synthesis that is apparently sufficient for the attainment of the critical cell size for START (Hanic-Joyce *et al.*, 1987a; Johnston & Singer, 1990). This suggests a mechanism for START regulation other than the minimum size requirement. It has been suggested that a labile protein(s) is required for cell cycle initiation at START (Shilo *et al.*, 1978b; Shilo *et al.*, 1979). It has been further suggested that a regulatory mechanism involving the *PRT1* gene product may be involved in the differential translation of this labile protein(s) in response to nutrient availability (Johnston & Singer, 1990).

It is possible that the *DNA26* gene may be involved in the regulation of the cell cycle via protein synthesis. Its influence on START may be exerted merely via the requirement for the attainment of a minimum size for cell cycle initiation. Alternatively the gene may have a more direct regulatory role in the differential translation of specific regulatory proteins that is normally dependent on nutrient availability. The severe apparent defect in protein synthesis caused by the *dna26-1* mutation begs its classification as a Class II

START mutation whilst the results obtained from the mating competency and heat shock experiments were inconclusive in this respect. Further experimentation to determine the conditions under which the *dna26-1* mutation causes a tighter arrest at START may be required before its strict classification as a Class I or Class II START mutation can be made. The study of further alleles of the *DNA26* gene can also be expected to contribute to the classification of the *dna26-1* mutation and the elucidation of the role of the *DNA26* gene in cell cycle control at START.

CHAPTER 4 : MOLECULAR CLONING OF THE *DNA26* GENE

4.1 INTRODUCTION

The development of cloning vectors and transformation procedures for yeast has made possible the application of recombinant DNA technology to the study of yeast genes (Hinnen *et al.*, 1978; Beggs, 1978). Most yeast vectors have been constructed as bacterial-yeast shuttle plasmids (for a review see Parent *et al.*, 1985). These vectors contain origins of replication and marker genes from both yeast and bacteria. This permits the amplification of vector DNA in bacteria (*E. coli*) and its easy isolation and purification in high quantities. The eukaryotic genes contained on the plasmid can then be expressed in yeast cells by transformation.

A common method for cloning genes from yeast is by selecting a wild type gene from a gene library by functional complementation of its mutant allele. Plasmid-borne gene libraries are constructed by the insertion of wild type yeast genomic fragments into an appropriate vector. The genomic DNA is usually fragmented by partial digestion with a restriction enzyme, usually *Sau*3A which has a tetranucleotide recognition sequence and generates free ends that are compatible with those generated by *Bam*HI digestion. The fragments generated by partial digestion are often fractionated for size by zonal centrifugation in a sucrose gradient. Fragments of the chosen size (for example 10-15kb) are then inserted into an appropriate vector; the insertion of partial *Sau*3A fragments into the *Bam*HI site of a shuttle vector is often detected by insertional inactivation of the tetracycline resistance gene carried by that vector.

After transformation of the appropriate mutant yeast strain and isolation of a library plasmid, the relevant gene must be located

within the cloned genomic fragment. This is usually carried out by deletion analysis using restriction enzymes. By this method various restriction fragments from the genomic insert are ligated into vectors which are used to retransform the original mutant yeast strain in a check for complementing activity. An alternative method for locating specific genes within a cloned genomic fragment is the disruption of that fragment at various sites by the insertion of bacterial transposons (transposon mutagenesis). Transposons are mobile genetic elements that can move from one site in the prokaryotic genome to another or to an autonomously replicating plasmid in a non-homologous recombination event (reviewed by Kleckner, 1977). Several transposons have been employed for the location of yeast genes within a cloned sequence, including Tn10 (Huisman *et al.*, 1987), Tn3 (Seifert *et al.*, 1986) and Tn5.

Tn5 (which was used in this study) has several beneficial characteristics for use in transposon mutagenesis. Most importantly it is highly non-specific for its sites of integration (Berg *et al.*, 1983) and undergoes transposition at an intermediate frequency compared with other transposons (Reznikoff, 1982). This means that it can transpose at a convenient frequency into many different sites in a target plasmid, but is relatively stable once insertion has occurred. Tn5 contains a kanamycin resistance marker which makes it easily selectable in *E. coli*, although this aminoglycoside phosphotransferase gene cannot normally confer G418 resistance on *S. cerevisiae* cells (Thiry-Blaise & Loppes, 1988). Standard protocols for mutagenesis of cloned genes using Tn5 have been reported (De Bruijn & Lupski, 1984) and Tn5 has been physically well characterized (Auerswald *et al.*, 1980). Tn5 insertions into bacterial-yeast

shuttle vectors have been shown to be stable when the mutagenized plasmid is used to transform yeast cells (Gordenin *et al.*, 1988). Moreover, physical mapping of a yeast gene by Tn5-mediated mutagenesis has been reported previously (Cannon & Tatchell, 1987; Van Dyk *et al.*, 1986).

Further molecular biological techniques have been developed that complement and extend the classical approaches to the study of yeast genetics thereby increasing the incentive for the cloning of yeast genes (Struhl, 1983). *S. cerevisiae and Schizosaccharomyces pombe* (*S. pombe*) are the two yeasts in which such molecular techniques have been most developed. For instance, a cloned gene from either of these organisms can be targetted to its homologous site in the yeast genome by the processes of transformation and site-directed integration by homologous recombination (Orr-Weaver *et al.*, 1981; 1983). This technique combined with classical tetrad analysis can confirm the allelism of a cloned gene with its homologous genomic copy. A related transformation technique has also been reported which allows disruption of a targetted genomic sequence (Rothstein, 1983). Again this technique depends upon the prior cloning of the yeast gene of interest. The cloned sequence is disrupted in vitro and used to replace its homologous functional copy in the yeast genome. This technique allows the null phenotype for the gene of interest to be analysed, determining whether the gene is essential for cell viability.

The cloning of a yeast gene can facilitate its physical mapping to a yeast chromosome. Electrophoretic methods have recently been described that permit the physical isolation of the sixteen chromosomes of *S. cerevisiae* (Schwartz & Cantor, 1984; Vollrath *et*

al., 1988). These techniques are based upon the physical perturbations that occur in DNA molecules in the presence of an applied electric field. The time required for a DNA molecule to relax to its most stable conformation after removal of an electric field is dependent upon that molecule's molecular weight and is known as the viscoelastic relaxation time. Pulsed field gel electrophoresis is carried out by the application of two alternating electric fields applied at specific angles relative to the direction of molecular migration (reviewed by Lai, *et al.*, 1989). Separation of large DNA molecules by this system still depends on the sieving affect of the gel matrix as in normal gel electrophoresis. However the most crucial factor to the resolution of large DNA molecules is the time taken for the molecules to change conformation and reorientate to the direction of an electric field applied at an alternating angle.

The system of pulsed field gel electrophoresis using clamped homogenous electric fields (CHEF) ensures that an homogenous field is applied over the whole gel in which DNA molecules are being separated (Chu *et al.*, 1986). This ensures that straight lanes are obtained and molecules separate according to their molecular weight. Separated chromosomes can be transferred to nylon or nitrocellulose membranes and hybridization analysis carried out (Southern, 1975). When appropriately labelled, a cloned yeast gene can therefore be used as a DNA probe to reveal the chromosome on which its homologue is located. The cloned yeast gene can also be used further for fine genetic mapping of its chromosomal locus. The gene is inserted into an integration vector that also carries a yeast marker. Directed integration of the plasmid to the genomic locus of the cloned

sequence results in the marking of that locus with an easily scorable marker for genetic mapping (Orr-Weaver *et al.*, 1981).

The physical isolation of large quantities of a cloned DNA fragment permits the application of techniques for the determination of its nucleotide sequence (Sanger *et al.*, 1977). Sequence analysis of a gene can provide insight into the structure and function of its predicted gene product. Large databases of gene sequences from many different organisms have been compiled. Therefore, if the sequence of a novel gene displays homology with that of a previously characterized gene, a prediction of the function of the cloned gene's product can be made.

In vitro mutagenic techniques may be applied for the creation of specific mutations in cloned genes. The altered gene sequence may then be expressed in the presence of a disrupted copy of its genomic allele. Such mutagenic techniques can yield instructive information revealing critical domains or active sites in structural genes and important motifs in sequences controlling gene expression. The cloning of a gene can also facilitate its overexpression allowing purification of the protein product that it encodes. Antibodies can be raised against a purified protein thus allowing its immunoprecipitation from cell lysates. By this means elucidation of the biological activity of a protein and its association with other gene products can be attempted.

A good example of the potential benefits involved in the cloning of a gene which is also relevant to the present study is provided by the isolation of the *CDC28* gene of *S. cerevisiae*. Mutations in the *CDC28* gene were first identified as causing an arrest of the *S. cerevisiae*

cell cycle in G1 (Hartwell *et al.*, 1974; Reed, 1980). The *CDC28* gene was cloned by functional complementation of a temperature sensitive *cdc28* mutation (Nasmyth & Reed, 1980). The nucleotide sequence of the *CDC28* gene was subsequently determined and found to possess homology with vertebrate protein kinase genes (Ferguson *et al.*, 1986; Lorincz *et al.*, 1984).

Antibodies raised against regions of the Cdc28 protein precipitate a 34kd protein ($p34^{cdc28}$). This protein possesses a kinase activity that phosphorylates an endogenous *S. cerevisiae* 40kD protein (Reed *et al.*, 1985) and bovine histone H1 (Wittenberg & Reed, 1988) on serine and threonine residues. The p40 kinase activity in immunoprecipitates from *S. cerevisiae* cell extracts has been shown to be restricted to the early G1 phase of the cell cycle of actively proliferating cells. This kinase activity has also been demonstrated to be thermolabile in extracts from cells containing a temperature sensitive *cdc28* mutation (Mendenhall *et al.*, 1987). Immunoprecipitation of $p34^{cdc28}$ also permitted the study of the regulation of its kinase activity during the cell cycle. Thus regulation of $p34^{cdc28}$ is thought to be at least partly achieved through its association with and dissociation from other proteins (Wittenberg & Reed, 1988) and not by overall phosphorylation state of the protein itself (Mendenhall *et al.*, 1987). A structural and functional homologue of the *CDC28* gene has been identified in a range of evolutionarily diverse eukaryotes including *S. pombe* (Beach *et al.*, 1982), humans (Lee & Nurse, 1987; Wittenberg & Reed, 1989) and higher plants (Feiler & Jacobs, 1990).

It was decided to clone the *DNA26* gene by functional complementation of the temperature sensitive *dna26-1* mutation using a plasmid-borne

library of wild type yeast genomic fragments. The aim was to subsequently use the cloned *DNA26* gene to deduce the location of homologous sequences in the *S. cerevisiae* genome. Further characterization of the *DNA26* gene was envisaged via gene disruption and gene expression experiments, nucleotide sequence analysis and isolation of the *DNA26* gene product.

4.2 MATERIALS AND METHODS

STRAINS

The strains of *S. cerevisiae* and *Escherichia coli* (*E. coli*) that were used during the cloning procedures are listed in Tables 4.1 and 4.2 respectively.

MEDIA

LB (Luria-Bertani) Medium

1%(w/v) bacteriological tryptone (Difco), 0.5%(w/v) yeast extract (Lab-M), 1%(w/v) sodium chloride, adjusted to pH7.5 with 1M NaOH. 2%(w/v) agar (Lab-M) was added to solid medium.

Antibiotics were added to a concentration of :

Ampicillin	35-50µg/ml from a 25mg/ml stock (bacterial selection)
Kanamycin	50µg/ml from a 25mg/ml stock (selection for transposon Tn5)
Chloramphenicol	170µg/ml from a 30 mg/ml stock (plasmid amplification)

The media used to grow yeasts, including YEPD and SD medium are described in Chapter 2, section 2.1.

BUFFERS AND SOLUTIONS

All solutions were prepared using MilliQ reagent grade water and autoclaved where appropriate. Chemicals were obtained from Sigma or BDH unless otherwise indicated.

Strain	Mating Type	Genotype	Source
JL448	a	*dna26-1 ade1 ade2 ura1 his7 lys2 tyr1 gal1*	L.D.
TDE/16A	a	*dna26-1 ade2 trp1 ura3-251/373 leu2-3/112*	D.E.
MD40/4C	alpha	*trp1 ura2 his3-11/15 leu2-3/112*	M.T.
DBY746	alpha	*trp1*-289a *his3-1 leu2-3/112 ura3-52*	D.B.
DBY/JL/6A	a	*cdc28-D1 leu2-3/112 trp1 his3 his7 ade1 ade2 ura1 ura3 tyr1 lys2*	D.E.
DBY/28/27	alpha	*cdc28-6 leu2-3/112 his3 ura3 met8 tyr1 arg1*	D.E.
DBY/28/42	alpha	*cdc28-15 leu2-3/112*	D.E.
DBY/36/5	alpha	*cdc36-16 leu2-3/112 trp1 ura3 ura1*	D.E.
DBY/39/39	a	*cdc39-1 leu2-3/112 trp1 his3 met2 tyr1 cyh2*	D.E.
DBY/37/14	a	*cdc37-1 leu2-3/112 trp1 ura3 cyh2*	D.E.
YPH148	alpha	*trp1-1 his7 ade- lys2-801 ura3-52*	M.P.
YNN295	alpha	*trp1-1 his7 ade- lys2-801 ura3-52*	BIO.

TABLE 4.1 Yeast Strains Used in Molecular Studies

The genotypes of the strains employed during the molecular analyses are shown as well as the sources from which they were obtained. L.D., Lawrence Dumas; D.E., constructed by Author; M.T., Mick Tuite; M.P. - Mick Pocklington; D.B., David Botstein; BIO., Bio-Rad Laboratories Ltd.

Strain	Genotype	Source
HB101	F⁻, *hsdS20*(r_B^-,m_B^-), *recA13*, *ara14*, *proA2*, *lacY1*, *galK2*, *rpsL20*(Sm^r), *xyl-5*, *mtl-1*, *supE44*, *lambda*⁻	N.C.T.C.
DH5alpha	F⁻,*Ø80d*, *lacZΔM15*, *endA1*, *recA1*, *hsdR17*(r_K^-,m_K^+), *supE44*, *thi1*, *lambda*⁻, *gyrA*, *relA1*, *Δ(lacZYA⁻argF)*, *U169*	P.T.
PCT800	*supE*, *supF*, *hsdS*⁻, *met*⁻, *recA56*, *Tn5*	D.D.

TABLE 4.2 Strains of *E. coli* used in Molecular Work

The genotypes of the *E. coli* strains used in molecular analyses are shown as well as the sources from which they were obtained. *E. coli* cultures were routinely stored in 40%(v/v) glycerol at -20°C (referred to in the text as glycerol stock cultures). N.C.T.C., National Collection of Type Cultures; P.T., Paul Towner; D.D. - David Dymock.

TE Buffer

10mM Tris.HCl (pH7.6), 1mM ethylene diamine tetraethanoic acid (EDTA, pH8.0).

Solution-I

25mM Tris.HCl (pH8.0), 10mM EDTA (pH8.0), 50mM glucose.

Alkaline SDS Solution

0.2M NaOH, 1%(w/v) sodium dodecyl sulphate (SDS) - freshly prepared.

Solution-III

11.5ml of glacial acetic acid added to 60ml of 5M potassium acetate and 28.5ml of H_2O.

Solution-1 (10X Stock)

18g of glucose, 6.04g of Tris.base, 7.44g of EDTA titrated to pH8.0 with HCl and made up to 200ml. The solution was filter sterilized and stored at 4°C.

Solution-3

28.8ml of glacial acetic acid added to 80ml of H_2O on ice and the pH adjusted to 4.8 with 10M KOH.

STE Buffer

10mM NaCl, 10mM Tris.HCl (pH 7.5), 1.0mM EDTA (pH 8.5).

Phenol

Chromatography-grade phenol (BDH) was melted at 55°C and 8-hydroxyquinoline added to 0.1%(w/v). The phenol was equilibrated several times with STE buffer and stored at 4°C.

Phenol:chloroform

Phenol equilibrated with STE buffer was mixed in a 1:1 ratio with

chloroform by vigorous shaking and stored at 4°C.

SCE Buffer

1.0M sorbitol, 0.1M sodium citrate, 60mM EDTA (pH5.8).

GH Solution

4.5M guanidine hydrochloride, 150mM NaCl, 100mM EDTA (pH8.0), 0.05%(w/v) sodium N-lauryl sarcosine (Sigma 1-5125).

CAG Buffer

60mM $CaCl_2$, 15%(v/v) glycerol, 10mM PIPES (pH7.0).

EP Buffer

272mM sucrose, 10mM HEPES (pH7.4).

SOC

2%(w/v) Bactotryptone, 0.5%(w/v) yeast extract,, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose.

LA Buffer

0.1M lithium acetate in TE buffer.

YS

50%(v/v) YEPD in 1.0M sorbitol.

Universal Restriction Buffer

33mM Tris. acetate, 66mM potassium acetate, 10mM magnesium acetate, 4mM spermidine, 0.5mM dithiothreitol.

TBE Buffer

0.089M Tris.base, 0.089M boric acid, 0.002M EDTA.

TAE Buffer (50X Stock)

242g of Tris base, 57.1ml of glacial acetic acid, 100ml of 0.5M EDTA

pH8.0 and made up to 1l with H_2O.

Loading Buffer

0.25%(w/v) bromophenol blue, 0.25%(w/v) xylene cyanol, 30%(v/v) glycerol.

10X TM Buffer

700mM Tris.HCl pH7.6, 100mM $MgCl_2$.

TCM Buffer

10mM Tris.HCl pH7.5, 10mM $CaCl_2$, 10mM $MgCl_2$.

20X SSC

3M NaCl, 0.3M tri-sodium citrate, pH7.0

(Pre)hybridization Solution

5X SSC, 0.1%(w/v) sodium N-laurylsarcosine (Sigma L-5125), 0.02%(w/v) SDS, 1.5%(w/v) blocking reagent (BCL). Blocking reagent was dissolved by heating the solution in microwave oven until bubbles formed followed by stirring at 50-70°C for 45-60min.

Wash Solution-2

0.5X SSC, 0.1%(w/v) SDS

Buffer-2

1%(w/v) blocking reagent and 50μg/ml herring sperm DNA (Sigma) in Buffer-1. The herring sperm DNA was suspended in 1ml of Buffer-1, autoclaved at 15lb/in^2 for 5min and added to Buffer-2.

Phosphate/Glycerol Buffer

100ml of 0.1M sodium phosphate buffer pH7.6 was prepared by adding 43.5ml of 0.2M Na_2HPO_4 to 6.5ml of 0.2M NaH_2PO_4 and making the volume up to 100ml with H_2O. 100ml of the phosphate/glycerol buffer was

prepared by adding 10ml of 0.1M sodium phosphate buffer to 90ml of 50%(v/v) glycerol.

DNA EXTRACTION METHODS

i) Mini-Prep. from *E. coli* (after Maniatis *et al.*, 1982).

A single colony of *E. coli* was inoculated into 5ml of LB + ampicillin (LB+AMP) and grown to saturation overnight at 37°C with agitation. 1.5ml of culture were harvested by centrifuging for 1.5min at high speed in a microcentrifuge (MSE). The cells were washed in 1ml of TE buffer and the pellet resuspended in 100µl of solution-I. 200µl of alkaline SDS were added and the solution mixed by inversion of the microfuge tube. The tube was cooled on ice for 5min. 150µl of solution-III were added and the tube vortexed briefly before incubation on ice for a further 5min. The cell debris was pelleted by centrifugation at high speed for 3min. The supernatant was transferred to a fresh, sterile tube and extracted with an equal volume of phenol:chloroform. Two volumes of ethanol were added and the DNA precipitated at -70°C for 30min. The DNA was collected by centrifugation at high speed for 15min at room temperature followed by washing with 200µl of 70%(v/v) ethanol. The pellet was dried under vacuum for 10min and resuspended in 50µl of TE buffer.

ii) Midi-Prep. from *E. coli* (Leicester Biocentre Cloning Manual).

10ml of LB+AMP were inoculated with 200µl of a glycerol stock culture of *E. coli*. The culture was incubated overnight at 37°C with agitation. 500µl of the starter culture was inoculated into 80ml of LB+AMP and incubated overnight at 37°C with vigorous shaking. The cells were harvested by centrifugation at 11950g for 10min in a DuPont Sorvall RC-5C centrifuge at 4°C and resuspended in 1.6ml of

cold 1X solution-1 containing 2mg/ml lysozyme (Sigma L-6876). 3.2ml of alkaline SDS were added and the solution incubated on ice for 5min. 2.4ml of 3M Sodium acetate pH5.5 was added and the solution mixed by gentle swirling followed by incubation on ice for 1hr. The cell debris was removed by centrifugation at 11950g for 10min at 4°C. Two volumes of cold ethanol were added to the supernatant followed by incubation at -20°C for 20min. The DNA was collected by centrifugation at 11950g for 10min and dried under vacuum for 15min before resuspension in 720µl of TE buffer and transfer to two 1.5ml microcentrifuge tubes. 7.2µl of RNase (10mg/ml stock) were added and the solution incubated at 37°C for 30min. The solution was then extracted successively with an equal volume of phenol, phenol:chloroform and finally chloroform:isoamylalcohol (24:1). 3M sodium acetate was added to the final aqueous solution to a concentration of 0.3M followed by the addition of two total volumes of ethanol and incubation at -70°C for 30min. The DNA was recovered by centrifugation in a microcentrifuge at high speed for 15min, washed with 250µl of 70%(v/v) ethanol and dried under vacuum for 15min. The DNA was suspended in 300µl of TE buffer and stored at 4°C.

iii) Maxi-Prep. from *E. coli* (J.Griffiths, personal communication). 5ml of LB+AMP were inoculated with 200µl of a glycerol stock culture of *E. coli*. This starter culture was incubated overnight at 37°C with agitation after which 250µl were used to inoculate 50ml of LB+AMP which was incubated at 37°C with vigorous shaking until the culture had reached an optical density of $O.D._{610}$ of 0.6. 30ml of the growing culture were then transferred to 500ml of LB+AMP and growth was permitted to continue for 2.5hr before addition of

chloramphenicol to a final volume of 170µg/ml. The culture was incubated for a further 12-16 hours at 37°C. The cells were harvested in 250ml centrifuge pots by centrifugation at 16270g for 2min. The pellet was resuspended in 50ml of solution-1 containing Lysozyme at a concentration of 2mg/ml and incubated on ice for 5min. 100ml of alkaline SDS solution was added to the solution which was mixed well by swirling and incubated at room temperature for 5min. The cell debris was removed by centrifugation (23430g for 10min) and by passing the supernatant through glass wool. 0.6 volumes of propan-2-ol were added and the solution incubated on ice for 30min. The crude DNA sample was collected by centrifugation at 27500g for 10min and dried under vacuum for 15min. The pellet was resuspended in 10ml of TE buffer and extracted successively with an equal volume of phenol and phenol:chloroform and finally with two volumes of water saturated ether. 3M sodium acetate was added to a final concentration of 0.3M and two total volumes of ethanol were added. The DNA was precipitated at -70°C for 30min, collected by centrifugation at 11950g for 10min, vacuum dried and resuspended in 5ml of TE buffer. Caesium chloride was added to a final concentration of 1.15g/ml. 1.6ml of ethidium bromide (10mg/ml stock) were added followed by cooling at 4°C for 15min. The solution was centrifuged at 26890g for 10min. at 4°C and the supernatant transferred to a Beckman heat-sealable ultracentrifuge tube. The tube was filled to the neck with 1.0g/ml CsCl in TE buffer before being heat-sealed. Plasmid and chromosomal DNA were separated by isopycnic centrifugation in a Beckman L5-65 ultracentrifuge employing a VTi-65 rotor at 54krpm for 15hr at room temperature. The plasmid DNA band was visualized under U.V. illumination and transferred to a 15ml corex tube using a syringe. The sample was extracted 3-4 times

with an equal volume of water-saturated butanol. 5 volumes of water were then added and 2 total volumes of absolute ethanol. The DNA was precipitated at -70°C for 30min and collected by centrifugation at 11950g for 10min. The pellet was dried under vacuum and resuspended in 500µl of TE buffer. One further ethanol precipitation was carried out and the plasmid DNA resuspended in 300µl of TE buffer. DNA concentration was determined spectrophotometrically. 10µl of a plasmid DNA solution in TE was added to 990µl of H_2O in a quartz cuvette. The $O.D._{260}$ reading for the solution was recorded and the concentration of DNA calculated on the basis that a solution of DNA at a concentration of 50µg/ml has an $O.D._{260}$ of 1.0.

iv) DNA Extraction from *S. cerevisiae* (Holm *et al.*, 1986).

100ml of cells were grown to a cell density of $8X10^6$/ml at 30°C in selective, supplemented SD medium. The cells were harvested by centrifugation at 3150g for 10min in an MSE Centaur centrifuge and washed in 20ml of sterile H_2O. The final cell pellet was resuspended in 150µl of SCE buffer and transferred to a sterile 1.5ml microfuge tube. Dithiothreitol (0.5M aqueous stock) was added to a final concentration of 100mM and the suspension was incubated at room temperature for 20min. 10µl of a 10mg/ml stock of aqueous Lyticase (Sigma L-8012) was added to the suspension followed by incubation at 37°C for a further 20min or until sphaeroplasts had been generated as judged by microscopic analysis. 6µl of the cell suspension was placed on a slide and covered with a cover slip. 4µl of 10%(w/v) aqueous sarkosyl (sodium N-laurylsarcosine, Sigma L-5125) was added to the edge of the cover slip. Sphaeroplasts were recognized by their sudden darkening on contact with the sarkosyl. Sphaeroplasts

were harvested by centrifugation at high speed for 20secs in a microcentrifuge. The pellet was drained well, resuspended in 150µl of GH solution and incubated at 65°C for 10min. The solution was allowed to cool, followed by the addition of 150µl of cold absolute ethanol and centrifugation at high speed for 5min. The pellet was drained well and liquified by stirring with a micropipette tip for 3min followed by the gradual addition of 300µl of 10X TE buffer while stirring. 10µl of 5mg/ml RNaseA was added and the solution incubated for 45min at 65°C. The solution was extracted twice with 500µl of phenol:chloroform:isoamyl alcohol (24:24:1). 3M sodium acetate was added to the final aqueous phase to a concentration of 0.3M and two volumes of absolute ethanol were added. The DNA was precipitated at -70°C for 30min and collected by centrifugation at high speed for 15min. The pellet was vacuum dried and resuspended in 20µl of TE buffer.

TRANSFORMATION PROCEDURES

i) $CaCl_2$ Method for Transformation of *E. coli* (after Meyer *et al.*, 1977). A starter culture was prepared by inoculating 5ml of LB with 200µl of a glycerol stock culture of *E. coli* and incubating overnight at 37°C with agitation. 1ml of the starter culture was inoculated into 50ml of LB and the culture was incubated at 37°C with vigorous shaking until the optical density of the culture had reached an $O.D._{610}$ value of 0.5. The culture was placed on ice for 5min then washed twice with CAG buffer. After each wash the cells were harvested by centrifugation at 1912g for 4min at 4°C. The cells were resuspended in 20ml of ice-cold CAG buffer and incubated on ice for 1hr. Finally the cells were harvested at 4°C, resuspended in 5ml

of CAG buffer and incubated on ice for a further 30min. Cells that were excess to immediate requirements were stored at -20°C. 1-10µl of plasmid DNA were added to 100µl of competent cells and incubated on ice for 1hr. The cells were then placed suddenly into a water bath at 42°C for 2min. 1ml of pre-warmed LB was added and the suspension incubated at 37°C for 1-2hr. 100µl aliquots of the cell suspension were spread onto LB+AMP plates and incubated overnight at 37°C to allow colony formation.

ii) Transformation of *E. coli* by Electroporation (Dower *et al.*,1988). A volume of 5ml of LB was inoculated with 200µl of an *E. coli* glycerol stock culture and incubated overnight at 37°C with agitation. 2ml of the starter culture was used to inoculate 100ml of LB. The culture was incubated at 37°C with vigorous shaking until it had reached an $O.D._{610}$ value of 0.5. The cells were chilled on ice for 5min then harvested by centrifugation at 1912g for 8min at 4°C. The cells were washed once with 100ml ice-cold 1mM HEPES buffer, pH7.0 and twice with 6ml of 10%(v/v) glycerol. The cells were finally resuspended in 150µl of 10%(v/v) glycerol and placed on ice. 40µl of the cell suspension were transferred to a microfuge tube and 0.2µg of plasmid DNA added. After 60secs the cell suspension was transferred to a chilled electroporation cuvette (0.2cm electrode spacing) and a single electric pulse was applied (2.5kV, 25µF, T=6.7mSecs). The cells were immediately resuspended in 950µl of SOC and transferred to a sterile microfuge tube. After incubation for 1hr at 37°C, 100µl aliquots of the cell suspension were spread onto LB+AMP plates. The plates were incubated overnight at 37°C to allow colony formation.

iii) Lithium acetate method for transformation of *S. cerevisiae* (after Ito *et al.*, (1983)). Yeast cells were grown in 100ml of YEPD supplemented with adenine and uracil and incubated overnight at 25°C in a shaking water bath. When the culture had reached a cell density of $5X10^6$–$1X10^7$/ml the cells were harvested by centrifugation at 3150g for 10min and washed twice with 20ml of TE buffer. The cells were finally resuspended in 5ml of LA buffer and incubated at 25°C for 30min. The cells were then reharvested and resuspended in 0.5ml of LA buffer. 100µl aliquots of the cell suspension were each transferred to 1.5ml microfuge tubes on ice and approximately 2µg of plasmid DNA added. After a 10min incubation on ice, 1ml of aqueous 70%(w/v) polyethylene glycol 4000 (Fisons) was added and mixed with the cell suspension by repeated inversion of the tube. The cells were incubated at 25°C for 45min followed by a heat pulse at 45°C for 5min. The cells were harvested by centrifugation in a microcentrifuge at high speed for 45secs and resuspended in 0.5ml of YS. After incubation for 1hr at 25°C, 100µl aliquots of the cell suspension were spread onto selective SD plates. The plates were incubated at 25°C for 5–10 days to allow colony formation.

iv) Transformation of *S. cerevisiae* by Electroporation (after Hashimoto *et al.*, 1985). Yeast cells were grown in supplemented YEPD to a density of $1X10^7$/ml at 25°C in a shaking water bath. The cells were harvested at 3150g for 10min, washed with 20ml of EP buffer and finally resuspended in 2ml of EP buffer. 350µl aliquots were each transferred to a 1.5ml microfuge tube. 5–10µg (not more than 70µl) of plasmid DNA were added to each aliquot of cells followed by the addition of 420µl of 50%(w/v) polyethylene glycol 4000 in EP buffer. The suspension was incubated at room temperature for 1hr after which

time 0.8ml were transferred to a sterile electroporation cuvette (electrode separation 0.4cm). A single electric discharge was applied using a Gene pulser apparatus (Bio-Rad Laboratories Ltd.). The voltage applied was 5.25kV/cm using the 1.0μF capacitor (τ = 0.8-1.0msecs). The cells were transferred to a fresh sterile microfuge tube and incubated at room temperature for 60min prior to being harvested in a microcentrifuge at high speed for 25secs. The cells were resuspended in 1ml of EP buffer and 100μl aliquots were spread onto selective SD plates. The plates were incubated for 3-5 days at 25°C to allow colony formation.

PHENOTYPIC STABILITY DETERMINATION IN YEAST TRANSFORMANTS

To screen putative transformants for the presence of an autonomously replicating plasmid the stability of the phenotype conferred by the particular plasmid marker was assessed after growth of cells under non-selective conditions for that marker. Single transformant colonies were inoculated into 50ml of non-selective medium and grown to a cell density of $1X10^8$/ml at 25°C. A 100μl aliquot from each culture was serially diluted and spread onto non-selective plates to give a colony number of 50-100 per plate after 3 days of growth at 25°C. A total of 100 colonies from each culture were replica-plated or replica-patched to selective and non-selective plates and incubated for 3 days at 25°C. If plasmid-mediated complementation of a temperature sensitive mutation was being screened, replica-plating to one further non-selective plate was carried out. This plate was then incubated at 36°C for 4 days.

DIGESTION OF DNA WITH RESTRICTION ENDONUCLEASES

Restriction digests were carried out as described by Maniatis *et al.*, (1982). Up to 1μg of DNA was suspended in H_2O to a final volume of 18μl and 2μl of universal restriction buffer (McClelland *et al.*, 1988) or One-Phor-All buffer (Pharmacia LKB Biotechnology) added. 1-2μl of the appropriate restriction enzyme(s) were added and the reaction tube incubated at 37°C for 60-90min. If required, 1μl of 0.25M EDTA was added to stop the reaction. Restriction enzymes were obtained from Northumbria Biochemicals Ltd. (NBL) or Pharmacia LKB Biotechnology. Uncut lambda DNA (Sigma D-3654) was restricted in an identical fashion. Prior to use as a size standard in electrophoretic analyses, restricted lambda DNA was heated at 65°C for 5min to melt cohesive *"cos"* ends (Sanger *et al.*, 1982) and cooled on ice for a further 5 min. Standard curves for lambda restriction fragment sizes were drawn on semi-logarithmic graph paper.

AGAROSE GEL ELECTROPHORESIS

Electrophoretic analyses were carried out as described by Maniatis *et al.*, (1982). 3.5μl of loading buffer was added to 20μl restriction digest solutions. DNA fragment separation was carried out routinely using a mini-gel apparatus. A 0.8%(w/v) solution was prepared by melting agarose (Sigma A-6013) in running buffer (1X TBE). Ethidium bromide was added to a concentration of 1μg/ml prior to pouring the gel. Electrophoresis was carried out for 50 min using a Bio-Rad 3000Xi power pack set to deliver approximately 12V/cm of length of the gel at 150mA.

For more accurate analyses, large 12.5cm-length gels were used. The

appropriate percentage solution of molten agarose was prepared in running buffer (1X TAE). Electrophoresis was carried out for approximately 2.5hr with an applied voltage of 12V/cm at 150mA. The DNA was stained by soaking the gel in 0.5μg/ml ethidium bromide for 30-45min and destaining in 1mM $MgSO_4$ for 1hr. DNA bands were visualized using a short wave U.V. transilluminator (Fotodyne Incorporated).

CONSTRUCTION OF CIRCULAR RESTRICTION MAPS

Restriction fragment size data (in kb) was first used to calculate arc lengths using the equation :-

$$s = (f(c))/Kb$$

- where s is the arc length drawn for a linear DNA fragment of size f (kb), in a circular map of circumference c, for a plasmid of size Kb (in kb) and where $c = 2\pi r$.

The arc length data was then used to calculate the angle between adjacent restriction sites in a circular map using the equation :-

$$\theta' = (s(180))/(r\pi)$$

- where θ' is the angle between two restriction sites bordering a DNA fragment of arc length s, in a circular map of radius r. Maps were drawn using a compass and protractor.

Tn5-MEDIATED TRANSPOSON MUTAGENESIS

The procedure for Tn5-mediated mutagenesis of a multicopy plasmid was based on protocols described by De Bruijn & Lupski (1984) and Van Dyk *et al.*, (1986). *E. coli* strain PCT800 carrying a genomic copy of transposon Tn5, was transformed with plasmid p801 (see Figure 4.4).

A total of $5X10^3$ ampicillin resistant transformants were selected on 30 LB+AMP plates. For each plate, the ampicillin resistant colonies were resuspended in 3ml of LB. Each 3ml volume of suspended cells was pooled in a sterile 250ml flask. Ampicillin was added for plasmid selection and the culture incubated overnight at 37°C. 100µl aliquots of the undiluted culture were plated directly to 30 separate LB plates containing ampicillin and neomycin at concentrations of 50µg/ml and 250µg/ml respectively. The high concentration of neomycin in the above plates permits the selection of cells carrying multicopy plasmids with Tn5 insertions (Berg *et al.*, 1983). 500-1000 colonies per plate were obtained. The colonies from each plate were suspended in 3ml of LB and pooled in a sterile 250ml flask. The culture was incubated overnight at 37°C with selection for ampicillin and kanamycin resistance. The cells were harvested and DNA prepared by the midi-prep. method.

E. coli strain DH5alpha was transformed to kanamycin resistance with midi-prep. DNA (at a frequency of $6X10^2$ transformants/µg of DNA). 150 kanamycin resistant transformants were obtained and patched to LB plates containing ampicillin and kanamycin. Physical analysis of the plasmids carried by individual transformants was carried out by restriction analysis of "mini-prep. DNA". Transformant strains carrying plasmids that were used in the restriction analyses were stored separately in glycerol at -20°C.

LIGATION OF DNA FRAGMENTS

2.5µg of vector DNA was linearized by restriction digestion with *Cla*I for 90min at 37°C. The digestion volume was made up to 50µl with universal digestion buffer. The vector DNA was then

dephosphorylated by two consecutive 30min incubations with 3.5µl of calf intestinal alkaline phosphatase (a gift from Lynn Stanbridge) at 37°C. The DNA solution was then extracted with an equal volume of phenol, phenol:chloroform and isoamylalcohol, ethanol precipitated and resuspended in 5µl of TE buffer.

The insert DNA, for each ligation performed, comprised a 3kb *ClaI* fragment from the plasmid p801 (see Figure 4.4). 5µg of p801 DNA were digested with *ClaI*. The digest was loaded onto a 1%(w/v) agarose gel containing ethidium bromide at a concentration of 0.5µg/ml for the electrophoretic separation of restriction fragments. Ultra pure Low Melting Point agarose was used (a gift from Nigel Stenner). The 3kb *ClaI* fragment from p801 was removed from the gel to a sterile microfuge tube. The enveloping agarose was melted at 65°C and kept molten at 42°C during sampling. A 20µl ligation mixture was prepared consisting of 7µl of H_2O, 2µl of 10X TM buffer, 2µl of 0.1M DTT, 2µl of 10mM ATP (Sigma), 5µl of dephosphorylated vector DNA, 5µl of molten agarose / insert DNA and 2µl of T4 DNA ligase (NBL). The ligation reaction was carried out overnight at 15°C.

For transformation of *E. coli*, 40µl of TCM buffer was added to the ligation mixture which was then incubated at 65°C for 5min. 30µl of the mixture was transferred to a sterile microfuge tube on ice and used to transform 200µl of competent cells by the usual $CaCl_2$ method. A ligation mixture containing dephosphorylated vector as the only DNA present was used as a control in each ligation procedure.

ELECTROPHORESIS BY CLAMPED HOMOGENOUS ELECTRIC FIELDS (CHEF)

Separation of yeast chromosomes by electrophoresis was carried out

using the CHEF-DR™II pulsed field gel electophoresis system (Bio-Rad Laboratories).

Preparation of Yeast Chromosomes for CHEF Electrophoresis

Cells of strain YPH148 were prepared essentially as described by Schwartz & Cantor (1984) and outlined in the CHEF-DR™II Instruction Manual. 200ml of cells were grown to a density of 2 X 10^8/ml in YEPD at 30°C. The cells were harvested when still fresh in stationary phase by centrifugation at 3150g for 10min. The cells were resuspended in 3ml of phosphate-glycerol buffer. Cell wall digestion was carried out by adding 100µl of 2mg/ml Lyticase (Sigma, L-8012) dissolved in phosphate-glycerol buffer to 300µl of the cell suspension and incubating at 37°C for 20min. 300µl of the cell suspension was mixed with 900µl of ultra-pure low melting point agarose in 0.125M EDTA at a temperature of 50°C. The mixture was pipetted into casting wells and allowed to cool at 4°C for 20min. The agarose plugs were then treated with the series of washes described in the CHEF-DR™II Instruction Manual. Protease K obtained from Sigma (P-0390) was used during the deproteinization of the chromosomal preparations.

Conditions for Separation of Yeast Chromosomes by CHEF

1%(w/v) agarose gels were prepared using Ultra-Pure Agarose (BRL, 5510UA). Routine conditions for yeast chromosome separation were those recommended by the manufacturer (CHEF-DR™II Instruction Manual and Applications Guide) involving a 2 step separation (switch time 60secs for 15hr followed by a switch time of 90secs for 9hrs, both at a voltage of 200V). A Grant cooling unit was used to maintain the electrophoresis buffer at a temperature of 14°C. Where the separation of specific doublet bands was required, a single-step,

40hr separation was carried out at 23°C and 175V with a switch time of 75secs (Clive Edmonds, personal communication).

HYBRIDIZATION ANALYSES

The procedures of Southern blotting, probe production, DNA-DNA hybridization and detection were carried out essentially as described in "DNA Labelling and Detection - Nonradioactive", published by Molecular Biology Boehringer Mannheim (BCL). Solutions whose composition was modified from the manufacturer's protocol are mentioned in the text below and described in the Buffers and Solutions section of this chapter.

Southern Blotting

Southern transfer was carried out essentially as described by Southern (1975). Electrophoretically separated DNA fragments were depurinated by incubation of agarose gels in 0.25M HCl for 10min. 20X SSC was used for transfer of chromosomal sized DNA fragments to nylon membranes whilst 10X SSC was used as transfer buffer for smaller DNA fragments. DNA was transferred to Hybond-N nylon membranes (Amersham International Plc.) and cross-linked to the membrane by exposure to 1mW/cm^2 of U.V. radiation for 3min. Corresponding chromosomal bands were identified by measuring their migration distance from the loading well, firstly on the gel and subsequently on the membrane (prior to photography in each case).

Probe Production

Non-radioactive DNA probes were prepared according to the manufacturer's instructions. Digoxigenin-labelled DNA was

resuspended in 50µl of 0.1%(w/v) SDS in TE buffer by vigorous vortexing and stored at 4°C.

Hybridization Conditions and Detection of Probe

Membranes were prehybridized in 50ml of prehybridization solution at 65°C for at least 90min in a Hybaid rotary hybridization oven. The prehybridization solution was then replaced with 7.5ml of hybridization solution containing approximately 50ng of probe DNA. Hybridization was carried out at 65°C overnight (15hr). Prior to the detection of probe DNA the filters were washed twice in 100ml of Wash solution-1 and twice in Wash solution-2.

Detection of probe DNA was carried out immediately after the post-hybridization washes and according to the manufacturer's instructions. Buffer-2 was modified to contain herring sperm DNA in addition to blocking reagent. Washes were carried out in plastic sandwich boxes and the colour reaction in a plastic box closely matching the size of the membrane.

4.3 RESULTS

TRANSFORMATION OF TDE/16A

As the first step towards the isolation of the wild type *DNA26* gene by functional complementation of the *dna26-1* mutation, transformation of the *dna26-1* mutant TDE/16A was attempted using two separate yeast genomic libraries. One library was contained in the 2*µm*, *LEU2* vector pMA3A (Appendix 1(B)) and the other in the *URA3*, *ARS1*, *CEN4* vector YCp50 (Appendix 1(A)). Transformation of yeast cells by the technique of sphaeroplast transformation (Hinnen *et al.*, 1978) or transformation of intact cells in the presence of alkali cations (Ito *et al.*, 1983) proved initially unsuccessful. Due to the availability of the relevant apparatus, the transformation of yeast cells was attempted by the alternative technique of electroporation. A Bio-Rad Gene Pulser apparatus was used for the electroporation experiments.

Transformation of Intact Yeast Cells By Electroporation

Intact yeast cells were transformed by a modification of the method described by Hashimoto *et al.* (1985). The latter authors have reported that the optimum efficiency of transformation by electroporation occurs when the applied electric field pulse results in the death of 50% of the cell population. An initial experiment was therefore carried out to establish the conditions of electroporation under which 50% cell death of a population of TDE/16A cells would occur.

A range of voltage conditions were tested whilst using the 1µF capacitor of the Gene Pulser equipment. An applied voltage of 5.25kV/cm across the electrodes of a 0.4cm electroporation cuvette

resulted in approximately 50% cell death when a single electrical discharge was applied (Figure 4.1). These parameters were therefore chosen for the transformation of intact yeast cells by electroporation.

The *LEU2*, 2μm plasmid YEp13 (Appendix 1(C)) and the *S. cerevisiae* gene library constructed in YCp50 were employed as the transforming DNA for transformation of the *dna26-1* mutant TDE/16A. Plasmid DNA was prepared by the midi-prep. method and used to transform intact cells of TDE/16A in the presence of polyethylene glycol (PEG). Selection for transformants was made by screening for functional complementation of the *leu2-3/112* double mutation (YEp13) or the *ura3-251/373* double mutation (YCp50) in TDE/16A by the respective plasmid markers. Transformant colonies were counted after incubation for 10 days at 25°C. The transformants obtained with the YCp50 library DNA were screened for instability of an autonomously relicating plasmid (data not shown). Single transformant colonies were used to inoculate non-selective medium in which cultures were grown to stationary phase. In each culture approximately 20% of the cells lost the Ura$^+$ phenotype providing evidence that the transformed phenotype was due to the presence of the *URA3* marker on the autonomously replicating YCp50 plasmid.

A maximum transformation frequency of 21 transformants per μg of YEp13 DNA was achieved ($1.68X10^{-7}$ transformants/cell) whilst a frequency of 1.57 transformants per microgram of the YCp50 gene library DNA ($8.8X10^{-8}$/cell) was obtained (Table 4.3). Hashimoto *et al.*, (1985) reported a transformation frequency of approximately 90 transformants per μg of YEp13 DNA.

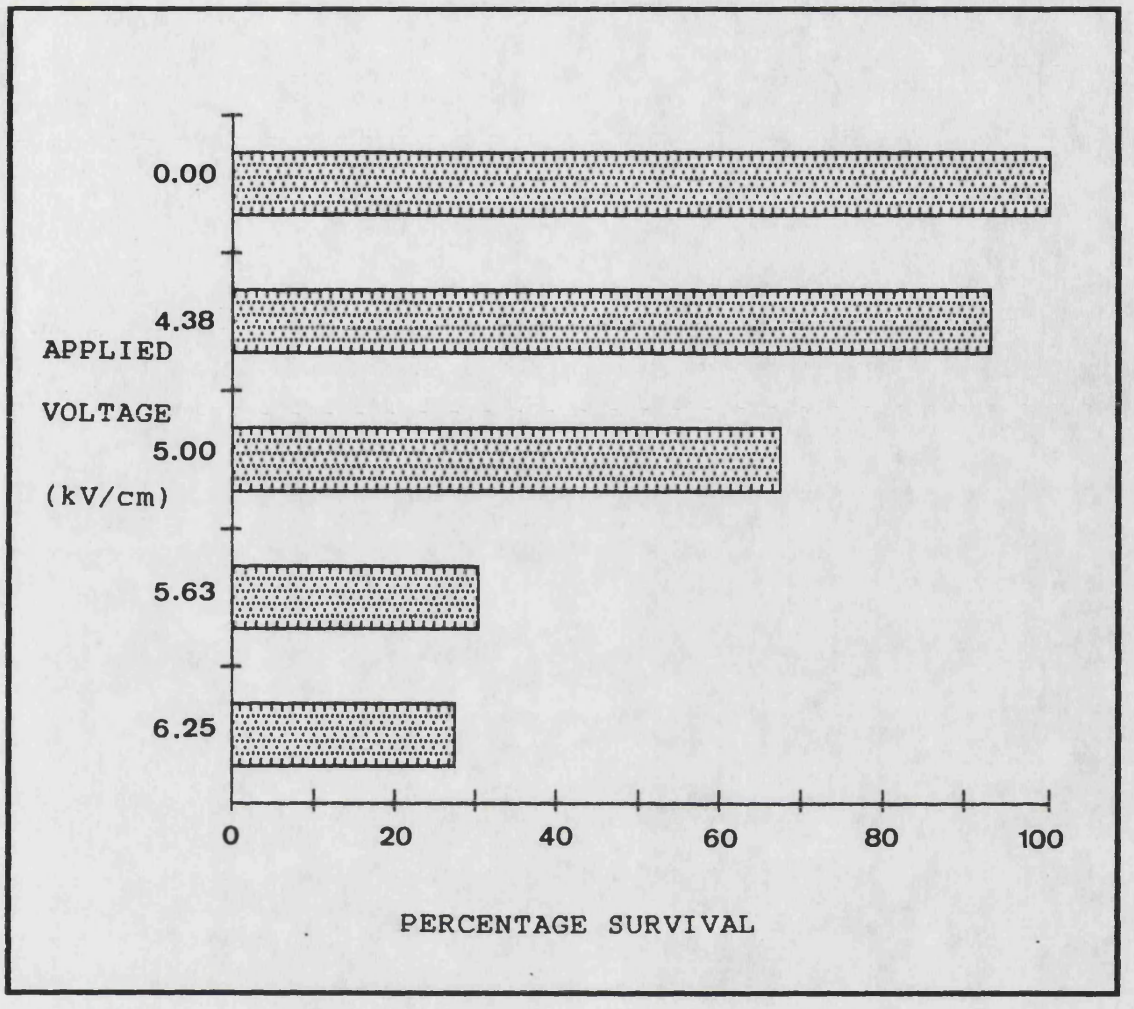


FIGURE 4.1 Measurement of Cell Death During Electroporation

Electroporation of intact cells of the *dna26-1* mutant TDE/16A was carried out at various voltages to establish the conditions for 50% cell death. The cells were prepared as described in Materials and Methods. A single electrical discharge was applied using the 1μF capacitor. Electoporation cuvettes with an electrode separation of 0.4cm were used. Viable cell counts were determined on supplemented SD plates after 4 days of incubation at 25°C.

PLASMID DNA	APPLIED VOLTAGE (kV/cm)	NUMBER OF TRANSFORMANTS	TRANSFORMANTS PER μg OF DNA
YEp13(7μg)	6.25	21	3.0
YEp13(7μg)	5.25	150	21.4
YCp50*(14μg)	5.25	11	1.57

TABLE 4.3 Efficiency of TDE/16A Transformation by Electroporation

The cells were transformed as described in section 4.2, Materials and Methods. Plasmid DNA was prepared by the midi-prep. method. Transformants were selected by complementation of the *leu2* (YEp13) or *ura3* (YCp50) mutation in TDE/16A on selective SD medium at 25°C. No transformants were obtained when TE buffer without plasmid DNA was added to the cells.

* Gene library constructed in YCp50 (Appendix 1(A)).

The equation devised by Clarke & Carbon (1976) was employed to calculate the number of transformants required to screen a yeast genomic library :-

$$N = \frac{\ln (1 - p)}{\ln (1 - f/g)}$$

- where N is the number of transformants to be screened, p is the probability of isolating a particular gene sequence, f is the size of the genomic inserts in the library vector and g is the size of the yeast genome (1.5×10^4 kb).

The yeast genomic library constructed in YCp50 consists of 10-15kb DNA fragments inserted at the *Bam*HI site of the plasmid. An average value for the genomic insert in YCp50 of 12.5kb was therefore inserted in the above equation leading to the calculation that a total of 2761 transformants would be required using this library to give a 90% chance of isolating a specific gene. As judged from the data in Table 4.3 it was estimated that approximately 1.75mg of plasmid DNA would be required to obtain 2761 transformants with the YCp50 gene library. This degree of transformation efficiency was considered to be too low to permit the isolation of the *DNA26* gene from the YCp50 library.

It is probable that further optimization of the conditions for electroporation would have yielded higher transformation frequencies. In fact conditions have been recently reported that can yield up to 4500 transformants per μg of plasmid DNA using the Gene Pulser apparatus (Delorme, 1989). However, transformation frequencies of about the latter level were achieved in parallel experiments in which a modification of the "lithium acetate method" was tested. This

method was therefore adopted for routine transformation of yeast cells.

Transformation of Intact Yeast Cells in the Presence of Lithium Ions

By the combination of various previously reported transformation conditions (Ito *et al.*, 1982; Bruschi *et al.*, 1987; Schiestl & Gietz, 1989) a protocol was devised for the transformation of intact cells of the *dna26-1* mutant TDE/16A. Various parameters in the initial protocol (essentially the protocol described in section 4.2, Materials and Methods) were modified to obtain an optimum frequency of TDE/16A cell transformation by the pMA3A-based gene library DNA. A range of concentrations for the final competent cell suspension were tested, each in the presence of 2.1μg of pMA3A gene library DNA and 44%(w/v) PEG. No saturation limit was reached for the cell concentrations tested. The highest competent cell concentration employed ($8X10^8$/ml) resulted in the highest number of Leu^+ transformants (Figure 4.2A).

Using the same batch of competent cells as for the experiment descibed above, the effect of PEG concentration on transformation efficiency was tested. A competent cell concentration of $4X10^8$/ml was employed to which 2.1μg of pMA3A gene library DNA and 1ml of PEG solution were added. The highest concentration of PEG employed (70%w/v) resulted in the highest transformation frequency (Figure 4.2B).

Using a second batch of competent TDE/16A cells the influence of i) pMA3A library plasmid concentration and ii) heat pulse temperature, was investigated. In the first experiment a competent cell concentration of $5X10^8$/ml was employed and a PEG concentration of

FIGURE 4.2 Optimization of the Conditions for Transformation of TDE/16A by the Lithium Acetate Method

The following four figures (A-D) describe variations in the conditions for transformation of the *dna26-1* mutant TDE/16A. Competent cells were transformed essentially as described in section 4.2, Materials and Methods. In each case an exponentially growing culture was harvested and competent cells resuspended to a final concentration of $5X10^8$/ml (except where indicated in A). 2.1μg of pMA3A library DNA were added to 100μl of competent cells; (in the experiment shown in Figure 4.2C the concentration of the plasmid stock suspension of pMA3A library was 0.07μg/μl and the various volumes containing 2, 4, 6, 8 and 10μg of DNA were made up to 200μl with TE buffer). 1ml of 44%(w/v) PEG 4000 was then added (except where indicated in B). The cells were heat shocked for 5mins at 42°C in a covered water bath (except where indicated in D). The number of transformants was scored on selective SD plates after 10 days of incubation at 25°C. The number of viable cells spread onto each transformation plate was estimated as $7.5X10^7$/ml by diluting and spreading 100μl aliquots of transformed cells to non-selective SD plates.

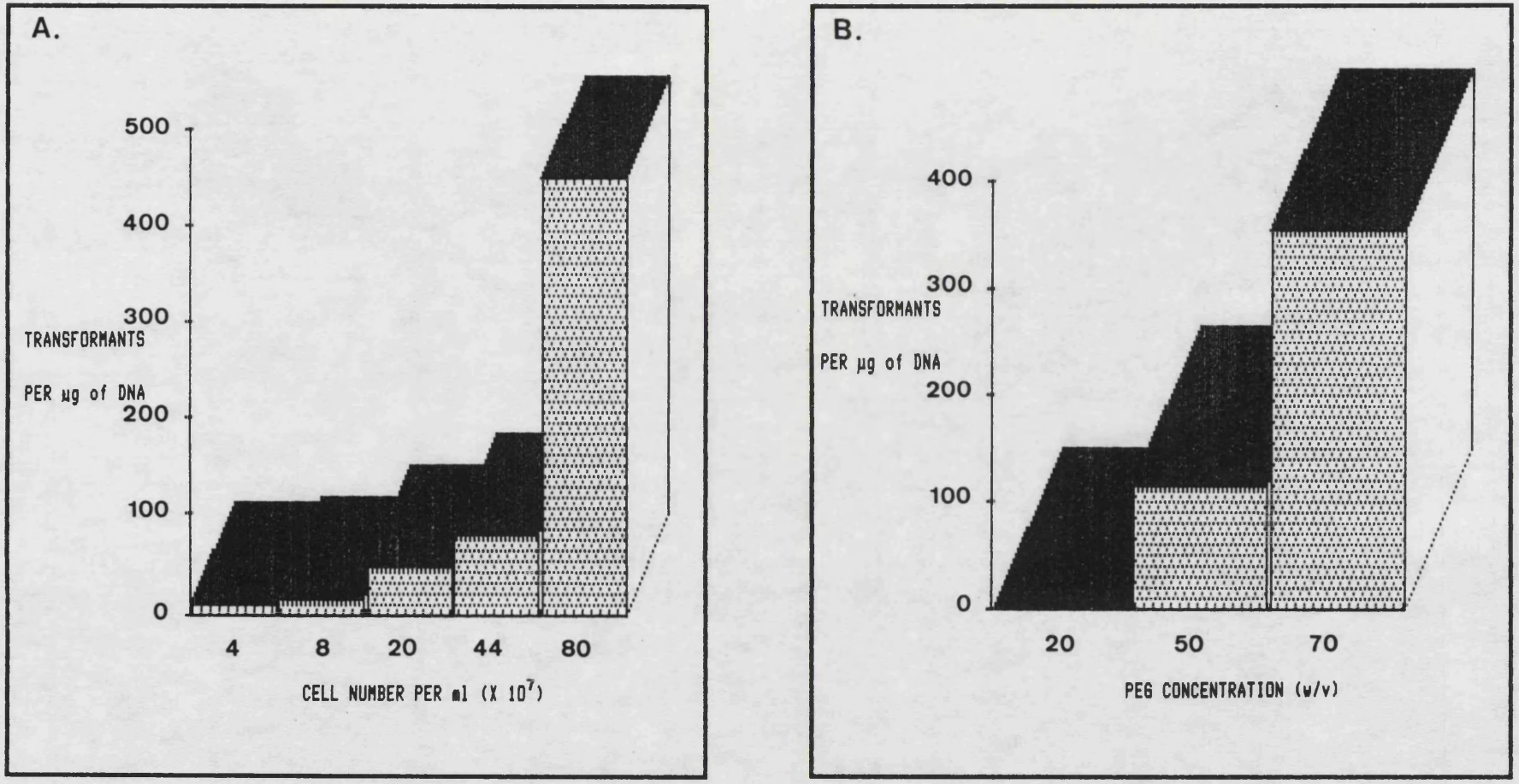


FIGURE 4.2 Transformation Efficiency of TDE/16A

A. Dependency on cell concentration.

B. Dependency on Polyethylene Glycol (PEG) concentration.

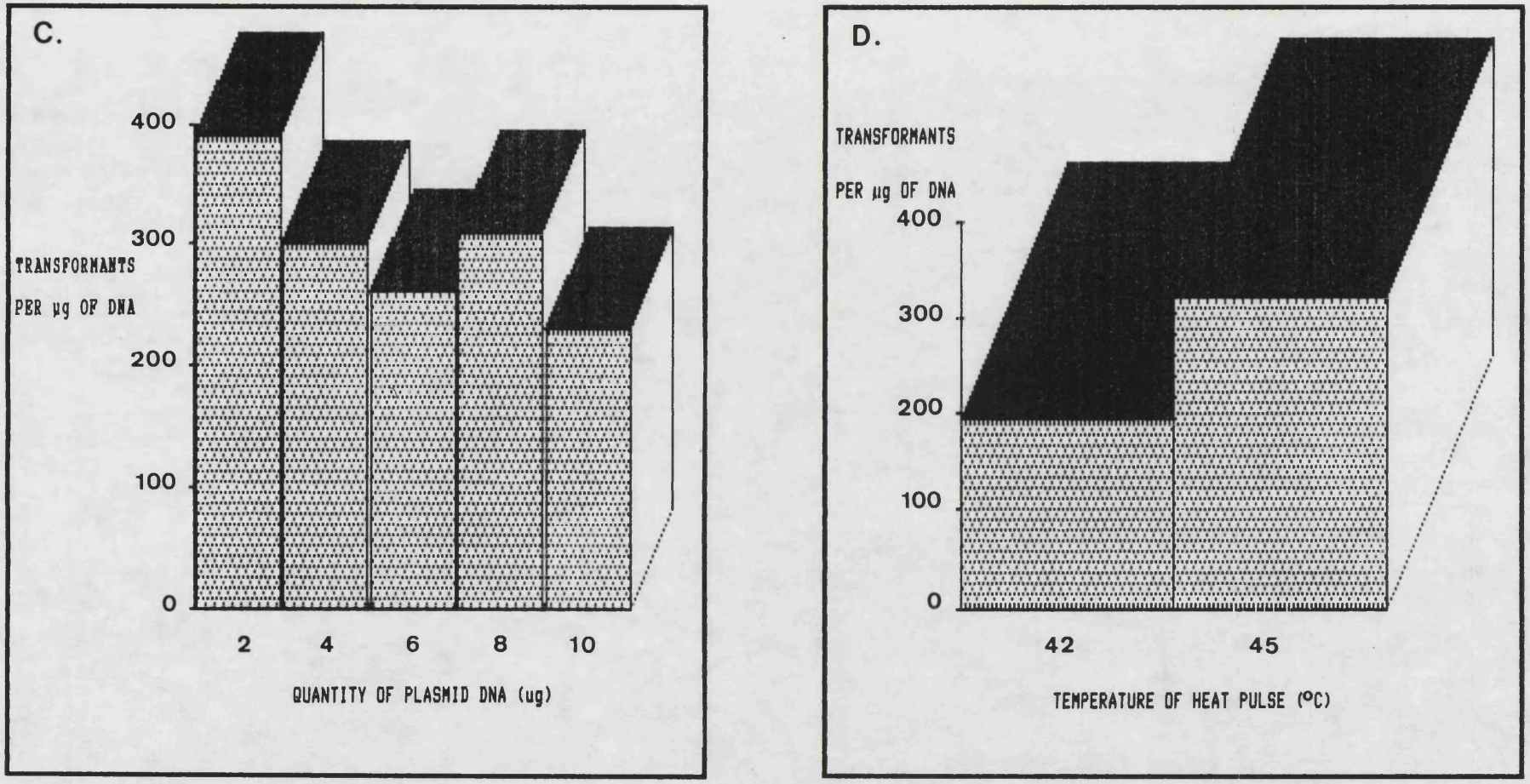


FIGURE 4.2 (Cont.) Transformation Efficiency of TDE/16A

C. Dependency on plasmid DNA quantity.

D. Dependency on heat pulse temperature.

44%(w/v). When different amounts of plasmid DNA were added (between 2 and 10μg) there was no observable increase in the transformation frequency beyond the addition of 2μg of plasmid DNA to 100μl of competent cells (Figure 4.2C). A 6.5-fold decrease in transformation frequency was also observed in this experiment when the cell suspension was vortexed in the presence of PEG (data not shown). The second experiment was carried out under similar conditions to the first. Varying the conditions of the heat pulse applied to the competent cells revealed an increase of transformation frequency of approximately 1.7-fold when the cells were heat shocked at 45°C compared with heat shock at 42°C (Figure 4.2D).

A transformation experiment was carried out employing all the optimum transformation conditions predicted by the above experiments. Transformation of TDE/16A by 3 different plasmids was tested (Table 4.4). The highest transformation frequency was obtained with YEp13 (2510 transformants/μg) whilst the maximum transformation frequency obtained with the pMA3A gene library was approximately 2-fold lower (1617 transformants / μg). The transformation frequency obtained with the YCp50 gene library was in the region of an order of magnitude lower (214 transformants/μg) than that obtained with the other plasmids used.

SCREENING THE GENE LIBRARIES CARRIED IN pMA3A AND YCp50

A total of 7500 Leu$^+$ colonies were obtained by transformation of the *dna26-1* mutant TDE/16A with the gene library constructed in plasmid pMA3A. The transformant colonies were first patched onto selective SD medium lacking leucine and incubated for 3 days at 25°C. These patches were then replica plated or replica patched onto two

TRANSFORMING DNA	VOLUME OF COMPETENT CELLS*	TRANSFORMANTS PER μg	TRANSFORMANTS PER CELL
pMA3A*	100	1175	$7.94X10^{-5}$
pMA3A*	200	1617	$1.04X10^{-4}$
YCp50**	100	214	ND
YEp13	100	2510	$1.36X10^{-4}$

TABLE 4.4 Transformation Frequency Conferred by Different Plasmids Under Optimized Conditions for TDE/16A

Cells of the *dna26-1* mutant TDE/16A were transformed as described in section 4.2, Materials and Methods. All plasmid DNA was prepared by the midi-prep. method. The number of transformants was scored on selective SD plates after incubation at 25°C for 10 days. The number of viable cells spread onto each transformation plate was calculated to be $7.4X10^{6}$ cells by serially diluting and spreading a cell sample onto non-selective SD medium.

* One 100μl aliquot consisted of $8X10^{7}$ cells suspended in LA buffer. In one sample the pMA3A library DNA was added to a 200μl aliquot of competent cells. The final volume of the cell suspension/DNA/PEG mixture in this sample was therefore increased by 100μl.

* pMA3A - genomic library constructed in pMA3A.

** YCp50 - genomic library constructed in YCp50.

ND - Not determined.

further selective SD plates. One of these plates was incubated at 25°C and the other at 36°C. Cell proliferation was scored after 4 days of incubation. A total of 12 out of 7500 patches displayed cell proliferation at both 25°C and 36°C. The transformants were each ascribed the prefix TMA and a number and included TMA5201, 2051, 5851, 1051, 2401, 4301, 6651, 4651, 801, 4101, 251 and 7551.

A total of 9000 Ura$^+$ colonies were obtained after transformation of TDE/16A with the gene library constructed in YCp50. The cells were replica patched to selective plates at 25°C and 36°C in an identical manner to that carried out with the pMA3A library except that the selective SD medium employed lacked uracil instead of leucine. A total of 2 out of the 9000 Ura$^+$ transformants displayed the ability to proliferate at both 25°C and 36°C. They were designated YCT1551C and YCT1816A.

Stability of the Transformed Phenotype

Each of the patches showing continued cell proliferation at 36°C maintained proliferation when streaked for single colonies on selective SD medium and incubated for 4 days at 36°C. To test whether the phenotype of the transformants was due to an autonomously replicating plasmid the mitotic stability of the Leu$^+$ or Ura$^+$ and the non-temperature sensitive (ts$^+$) phenotype was examined in each strain.

Plasmid stability experiments involving growth of the transformant strains under non-selective conditions were carried out. Experiments examining phenotypic stability during growth on selective medium were also carried out. Supplemented SD medium lacking either leucine (for the "TMA" transformants) or uracil (for the "YCT" transformants) was

used as the selective medium whilst non-selective SD medium contained all growth supplements required by the untransformed TDE/16A strain.

The results of the plasmid stability experiments are presented in Table 4.5. Five of the transformants derived from the transformation of TDE/16A with the pMA3A library displayed a high frequency of coincident loss of the Leu^+ and ts^+ phenotypes under non-selective conditions. These were transformants TMA4301, 2401, 2051, 801, and 4101. The data for these strains suggested that the transformed phenotype in each case was conferred by a mitotically unstable plasmid carrying a genomic fragment capable of suppressing the leucine dependent and temperature sensitive phenotype of TDE/16A. These transformants were therefore selected for further study.

Each of the transformants TMA1051, 7551, 251, 5851 and 4651 displayed a high frequency of loss of the ts^+ phenotype under non-selective conditions whilst simultaneously retaining the Leu^+ phenotype. The plasmids in these strains may have undergone specific rearrangement or deletion of the sequences responsible for suppression of the ts^- phenotype of TDE/16A. It is also possible that multiple plasmid species may have been present in these strains and that the particular species causing suppression of the ts^- phenotype was being preferentially lost. The plasmids in these transformants were not studied further. The transformants TMA5201 and TMA6651 showed a low rate of independent loss of either the Leu^+ or ts^+ phenotype or both. The plasmids in these strains may have also been undergoing rearrangement and were not included in further study.

Both YCT1816A and YCT1551C derived from transformation of TDE/16A with the gene library in YCp50 diplayed a very high frequency of loss

TABLE 4.5 Phenotypic Stability of TDE/16A Transformants

The data presented in Table 4.5, parts A and B, are for the number of colonies displaying a particular phenotype. Cultures were grown in medium that was selective (Sel.) or non-selective (Non-Sel.) for the plasmid marker and phenotypic stability was determined as decribed in section 4.2, Materials and Methods. The phenotypic determinants scored were leucine- (TMA transformants) or uracil- (YCT transformants) dependent growth and temperature-dependent growth at 36°C. 100 patched colonies were screened for each transformant under non-selective and selective conditions (except TMA4101 - 50 colonies on selective medium).

* Coincident loss of both the leucine independent (Leu^+) or uracil independent (Ura^+) and the non-temperature sensitive (ts^+) phenotype.

** Retention of both the Leu^+ or Ura^+ and the ts^+ phenotype.

\# Loss of the Leu^+ or Ura^+ phenotype and retention of the ts^+ phenotype.

\#\# Loss of the ts^+ phenotype and retention of the Leu^+ or Ura^+ phenotype.

TABLE 4.5A Phenotypic Stability of TDE/16A Transformants

STRAIN	GROWTH MEDIUM	COINCIDENT* LOSS	COINCIDENT** RETENTION	LOSS OF# Leu^+/Ura^+	LOSS OF## ts^+	INVIABLE
YCT1816A	Sel.	4	25	1	69	1
	Non-sel.	9	12	3	73	3
YCT1551C	Sel.	0	31	0	69	0
	Non-sel.	0	30	0	70	0
TMA801	Sel.	10	89	0	1	0
	Non-sel.	31	69	0	0	0
TMA4101	Sel.	12	37	0	1	0
	Non-sel.	33	67	0	0	0
TMA2051	Sel.	26	73	0	1	0
	Non-sel.	49	50	0	1	0
TMA2401	Sel.	3	81	0	0	16
	Non-sel.	46	37	0	0	17
TMA4301	Sel.	3	97	0	0	0
	Non-sel.	63	37	0	0	0

TABLE 4.5B Phenotypic Stability of TDE/16A Transformants

STRAIN	GROWTH MEDIUM	COINCIDENT* LOSS	COINCIDENT** RETENTION	LOSS OF# Leu$^+$	LOSS OF## ts$^+$	INVIABLE
TMA1051	Sel.	2	29	0	15	54
	Non-sel.	8	10	0	16	66
TMA7551	Sel.	3	88	0	5	4
	Non-sel.	54	35	2	7	2
TMA251	Sel.	1	5	0	94	0
	Non-sel.	33	7	0	59	1
TMA6651	Sel.	15	81	1	3	0
	Non-sel.	57	42	1	0	0
TMA4651	Sel.	9	64	0	27	0
	Non-sel.	32	50	0	18	0
TMA5851	Sel.	0	37	0	63	0
	Non-sel.	18	38	5	39	0
TMA5201	Sel.	11	88	0	1	0
	Non-sel.	22	78	0	0	0

of the ts$^+$ phenotype, even under selective conditions for the plasmid *URA3* marker. These plasmids may have undergone deletion of the sequences responsible for suppression of the ts$^-$ phenotype of TDE/16A. The high stability of the Ura$^+$ phenotype in YCT1551C suggested that the plasmid may have undergone integration into the genome of TDE/16A. This integration event may have disrupted the plasmid sequences responsible for suppression of the ts$^-$ phenotype.

Transformation of *E. coli*

The total DNA was extracted from all those yeast transformants that had shown cosegregation of the Leu$^+$ and ts$^+$ phenotypes during the mitotic stability experiments. DNA was also prepared from transformant TMA7551 which had displayed independent loss of the ts$^+$ phenotype at a low frequency. When competent cells of the *E. coli* strain DH5alpha were prepared by the usual treatment with $CaCl_2$ the yeast DNA failed to generate ampicillin resistant transformants. In an attempt to achieve a maximum efficiency of transformation the alternative method of transformation of DH5alpha by electroporation was adopted. This method was successful with a transformation frequency of between 100 and 1000 transformants per μg of yeast DNA being achieved for the DNA derived from transformants TMA4301, 2401, 2051, 801, 4101 and 7551. Selection in each case was for ampicillin resistance conferred by the *amp^R* gene resident in the pMA3A sequences of the library plasmids. The ability of the DNA prepared from the yeast transformants to transform *E. coli* to ampicillin resistance provided further evidence that the Leu$^+$/ ts$^+$ phenotype observed in these yeast strains was due to an autonomously replicating plasmid.

EcoRI Restriction Map of the Library Plasmids

Six ampicillin resistant colonies generated from DNA derived from the yeast transformant TMA801 were selected at random. DNA was prepared from these *E. coli* transformants by the mini-prep. method and digested with the restriction endonuclease *EcoRI*. The sizes of the restriction fragments generated were estimated by agarose gel electrophoresis and comparison with lambda DNA digested with *PstI*. This process was repeated for six *E. coli* transformants generated with DNA prepared from each of the other yeast transformants involved in this part of the study.

As seen in Table 4.6 the plasmids derived originally from the yeast transformants TMA801, 4301, 2401 4101 and 2051 each displayed a similar *EcoRI* restriction map. The only exception was for 4 *E. coli* transformants generated with DNA from the yeast strain TMA2051 for which only a single 4.4kb *EcoRI* band was detectable. This anomaly may have been due to the replication of multiple plasmid species in TMA2051 with only single species being recovered in independent transformants of *E. coli*.

It was not possible to determine from this analysis whether the plasmids from the above transformants contained exactly identical inserts. The mini-gel system employed for the restriction analyses was inefficient at detecting DNA fragments of molecular weight less than 0.8kb. The restriction data did suggest however that the plasmids derived from TMA801, 4101, 4301, 2051 and 2401 contained largely overlapping genomic inserts. The pMA3A library was constructed to contain 10-15kb-sized genomic inserts. Out of the 7500 transformants screened for this library the expected number of transformants carrying any particular genomic fragment was calculated

*Eco*RI FRAGMENT SIZE (kb)	YEAST TRANSFORMANT FROM WHICH PLASMID DNA WAS DERIVED					
	4101	2401	801	4301	2051	7551
0.6	ND	ND	+	ND	ND	+
0.7	+	ND	+	+	ND	+
0.8	-	+	+	+	+	+
1.6	+	+	+	+	+	-
2.7	+	+	+	+	+	+
4.2	+	+	+	+	+	+
4.4	+	+	+	+	+	-
~7.0	-	-	-	-	-	+

TABLE 4.6 *Eco*RI Restriction Fragments Generated from the Plasmids Carried by Five ts$^+$ Transformants of TDE/16A

The data represent restriction fragments generated by digestion of various plasmids with *Eco*RI. DNA was prepared from six *E. coli* transformants for each original yeast plasmid source and digested with *Eco*RI. Fragment size was determined by agarose gel electrophoresis. The data for plasmids derived from TMA4101, 2401, 801, 4301 and 7551 represent a result confirmed by six individual mini-preps. Only 2 *E. coli* transformants generated from DNA derived from TMA2051 contained plasmids that possessed the collection of *Eco*RI fragments shown in table. The other 4 transformants examined in this case displayed a single 4.3kb *Eco*RI-digested DNA fragment.

ND - Not detectable. The mini-gel system employed did not reliably detect all low molecular weight fragments at the DNA concentrations used.

to be approximately 3 (using the Clarke and Carbon equation - see above). The isolation of five plasmids containing an approximately identical region of the yeast genome therefore represented a fair approximation to the calculated frequency.

Six plasmids were also derived from independent *E. coli* transformants that had been generated with DNA isolated from TMA7551. Restriction of these plasmids with *Eco*RI generated some DNA fragments that were of identical size to those generated from the plasmids derived from the other transformant strains. Nevertheless the plasmids derived from TMA7551 lacked the 1.6kb and 4.4kb bands observed for the plasmids of the other transformants. Furthermore the plasmids derived from TMA7551 contained a novel 7.0kb *Eco*RI fragment. This suggested that the plasmids derived from TMA7551 contained genomic inserts possessing partially overlapping sequences with the inserts present in the plasmids from TMA801, 4101, 4301, 2401 and 2051.

Retransformation of the *dna26-1* Mutant with Library Plasmids

To check that the plasmids that had been isolated from the *E. coli* transformants could still cause suppression of the temperature sensitive mutation in the *dna26-1* mutant, they were used to separately retransform TDE/16A. Plasmids were prepared from single *E. coli* colonies by the midi-prep. method and attributed with a number corresponding to the number of the yeast transformant from which they were originally derived. Three plasmids p801, p4101 and p2401 that had appeared to be apparently identical by restriction mapping with *Eco*RI were prepared. Plasmid p7551 that had appeared to contain a distinct but overlapping genomic insert was also prepared and used to transform TDE/16A. The initial selection for

transformants was for leucine independence on selective SD medium at 25°C conferred by the *d-LEU2* marker on the library plasmid. 100 Leu$^+$ colonies derived from each plasmid source were replica patched onto pairs of selective SD plates. One of the pair of plates was incubated at 25°C and the other at 36°C. Cellular proliferation was scored after 4 days of incubation at the respective temperatures.

Plasmids p801, p4101 and p2401 each transformed TDE/16A at high frequency (858, 755 and 862 transformants per μg of plasmid DNA respectively) restoring both the Leu$^+$ and ts$^+$ phenotypes (Figure 4.3). This provided confirmation that these plasmids were responsible for the phenotype observed in the original yeast transformants. This result contrasted with that obtained for p7551. Although p7551 retransformed TDE/16A to leucine independence at a high frquency (586 transformants/μg) it failed to restore the ts$^+$ phenotype that had been observed in the original yeast transformant. The reason for this was unclear although it seemed possible from the *Eco*RI digestion data that p7551 might contain a truncated version of the gene responsible for the suppression of the *dna26-1* mutant phenotype.

The auxotrophic requirements of TDE/16A transformants containing plasmid p801, p2401 or p4101 were analysed on dropout medium. All of the transformants displayed an Ade$^-$ Trp$^-$ Ura$^-$ Leu$^+$ phenotype. This provided evidence that the gene on the genomic insert in these plasmids responsible for suppression of the *dna26-1* mutation was not tightly linked to the *ADE2*, *TRP1* or *URA3* locus.

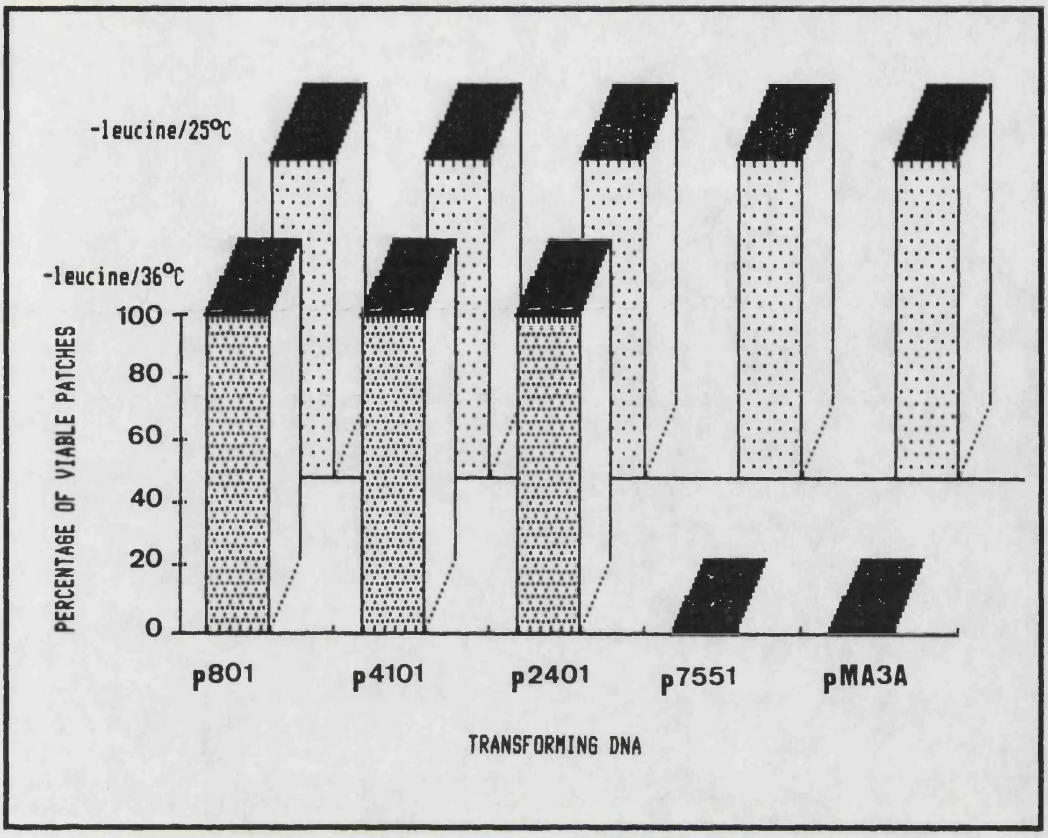


FIGURE 4.3 Phenotype of TDE/16A After Transformation with Different Plasmids

TDE/16A was transformed with the independently isolated library plasmids p801, p4101, p2401 and p7551 and the vector pMA3A. The viability of transformant colonies that had been replica patched to plates lacking leucine at 25°C (background) and plates lacking leucine at 36°C (foreground) was scored after 4 days at the respective incubation temperatures. Positive or negative proliferation was scored by comparison with patches of the strain TDE/6B transformed with pMA3A (data not shown). TDE/6B displays a ts^+ phenotype at 36° C and when transformed with pMA3A is capable of growth on medium lacking leucine.

Transformation of other START Mutants

Plasmid p801 was used to transform a number of mutant yeast strains including DBY/JL/6A(*cdc28-D1*), DBY/28/27(*cdc28-6*), DBY/28/42(*cdc28-15*), DBY/39/39(*cdc39-1*), DBY/36/5(*cdc36-16*) and DBY/37/14(*cdc37-1*). All of the strains contained a temperature sensitive START mutation and a *leu2-3/112* double mutation allowing the selection of transformants carrying plasmid p801. p801 transformed these strains to a Leu$^+$ phenotype at 25°C with frequencies varying between 10^1 and 10^3 transformants per μg of plasmid DNA (data not shown). No transformants were obtained for any of these strains when colony formation on transformation plates was directly screened at 36°C. Furthermore, no Leu$^+$ colonies obtained for these strains at 25°C were able to proliferate when streaked for single colonies on selective medium at 36°C. These results suggested that the cloned fragment in p801 did not contain the wild type *CDC28, CDC36, CDC39* or *CDC37* gene or a dosage suppressor of mutations in these genes.

RESTRICTION MAPPING OF THE CLONED SEQUENCES

One of the library plasmids capable of causing suppression of the *dna26-1* mutant phenotype, plasmid p801, was chosen for detailed restriction mapping. p801 DNA was prepared by the maxi-prep. method and purified by isopycnic centrifugation in a CsCl gradient. Large 15cm-length gels were used for the accurate sizing of restriction fragments. The sizes of the higher molecular weight DNA fragments (>3.0kb) were determined on 0.7% agarose gels whilst the sizes of lower molecular weight fragments were measured on 1.3% gels. Restriction fragment sizes were determined from a standard curve constructed by running a lambda ladder of known DNA fragment sizes on

the same gel as the restricted plasmid DNA.

The restriction analysis of p801 was carried out in three consecutive stages. Firstly, both p801 and pMA3A were digested with single restriction enzymes (Appendix 2(A)). This initial analysis identified the total number of sites in p801 for a particular restriction enzyme and the restriction fragments in p801 that were derived from pMA3A vector sequences. Where the restriction enzyme employed had only 1 or 2 sites which were located in the genomic insert of p801, it was possible to determine the approximate coordinates of those sites.

More accurate mapping of the restriction sites in p801 was achieved by carrying out simultaneous digests with two restriction enzymes (Appendix 2(B). Double digests tended to generate smaller DNA fragments the size of which were more accurately determined by the system employed. Finally, where the coordinates of restriction sites could not be accurately determined from double digests, p801 DNA was simultaneously digested with three restriction enzymes (Appendix 2(C). An example of the restriction analyses carried out is shown in Plates 4.1 and 4.2 which show the restriction fragments generated during double digests of p801 involving *Eco*RI in combination with a range of other restriction enymes.

The restriction map of plasmid p801 was determined from the above data (Figure 4.4). The plasmid was determined to be 14.76kb in size, containing a genomic insert into pMA3A that was approximately 6.9kb in size. The insert was therefore smaller than that expected for a library that was constructed to contain 10-15kb sized genomic fragments in pMA3A. The library was constructed by the insertion of

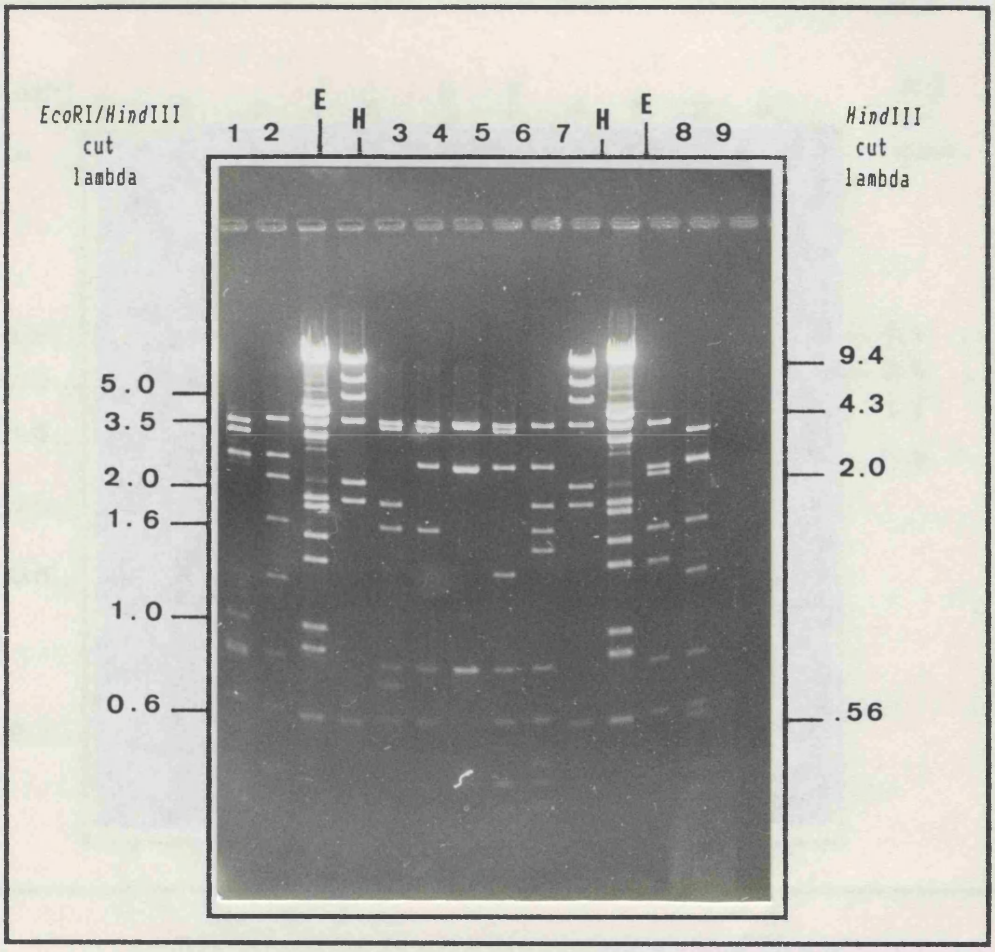


PLATE 4.1 Restriction Analysis of Plasmid p8O1

The above is an example of a series of "double digests" employing *Eco*RI in combination with one other enzyme. The above analysis was carried out in 0.7% agarose for accurate sizing of the higher molecular weight DNA fragments.

1. p8O1(*Eco*RI/*Pst*I)
2. p8O1(*Eco*RI/*Kpn*I)
3. p8O1(*Eco*RI/*Xba*I)
4. p8O1(*Eco*RI)
5. pMA3A(*Eco*RI)
6. p8O1(*Eco*RI/*Bam*HI)
7. p8O1(*Eco*RI/*Bgl*II)
8. p8O1(*Eco*RI/*Hind*III)
9. p8O1(*Eco*RI/*Sal*I)

E. lambda(*Eco*RI/*Hind*III)

H. lambda(*Hind*III)

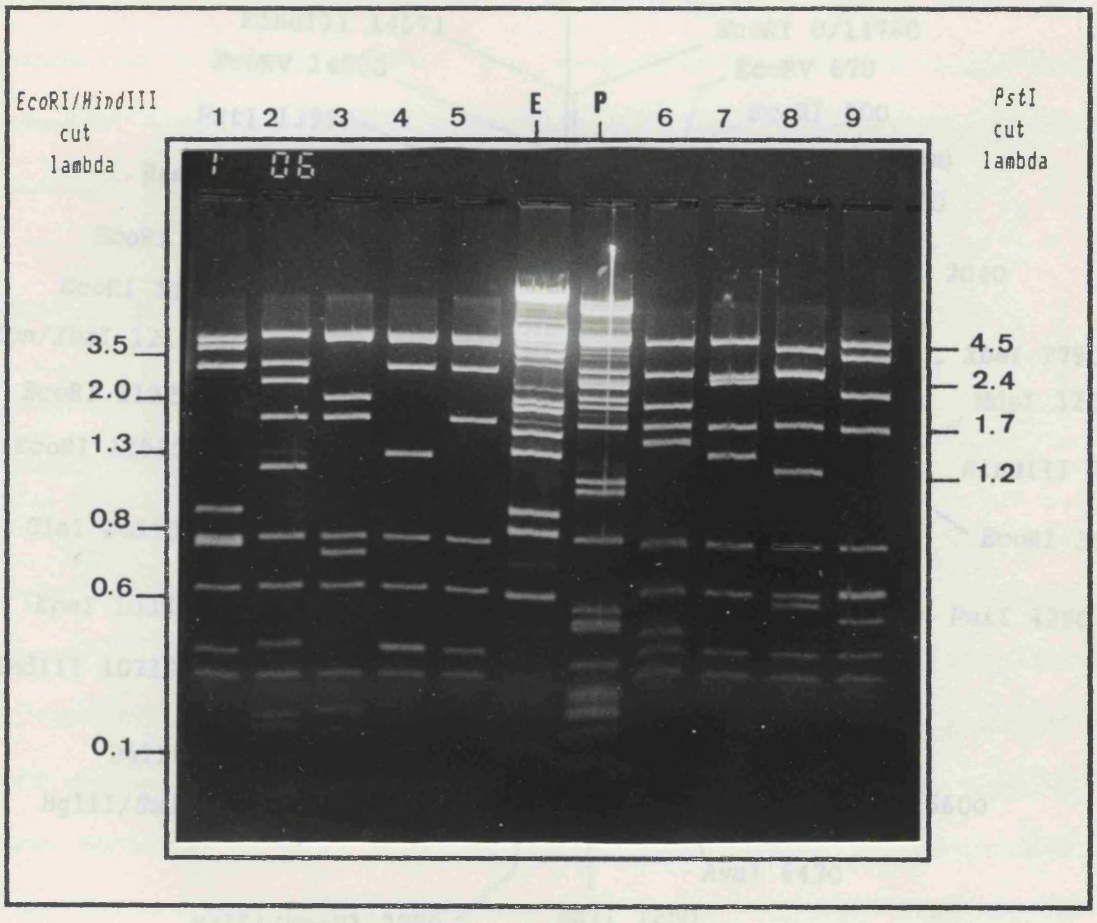


PLATE 4.2 Restriction Analysis of Plasmid p801

The above is an example of a series of "double digests" employing EcoRI in combination with one other enzyme. The above analysis was carried out in a 1.3% agarose gel for accurate sizing of the lower molecular weight DNA fragments.

1. p801(*EcoRI/PstI*)
2. p801(*EcoRI/KpnI*)
3. p801(*EcoRI /XbaI*)
4. p801(EcoRI/BamHI)
5. p801(*EcoRI*)
6. p801(*EcoRI/BglII*)
7. p801(*EcoRI/HindIII*)
8. p801(*EcoRI/SalI*)
9. p801(*EcoRI/ClaI*)

E. lambda(*EcoRI/HindIII*)

P. lambda(*PstI*)

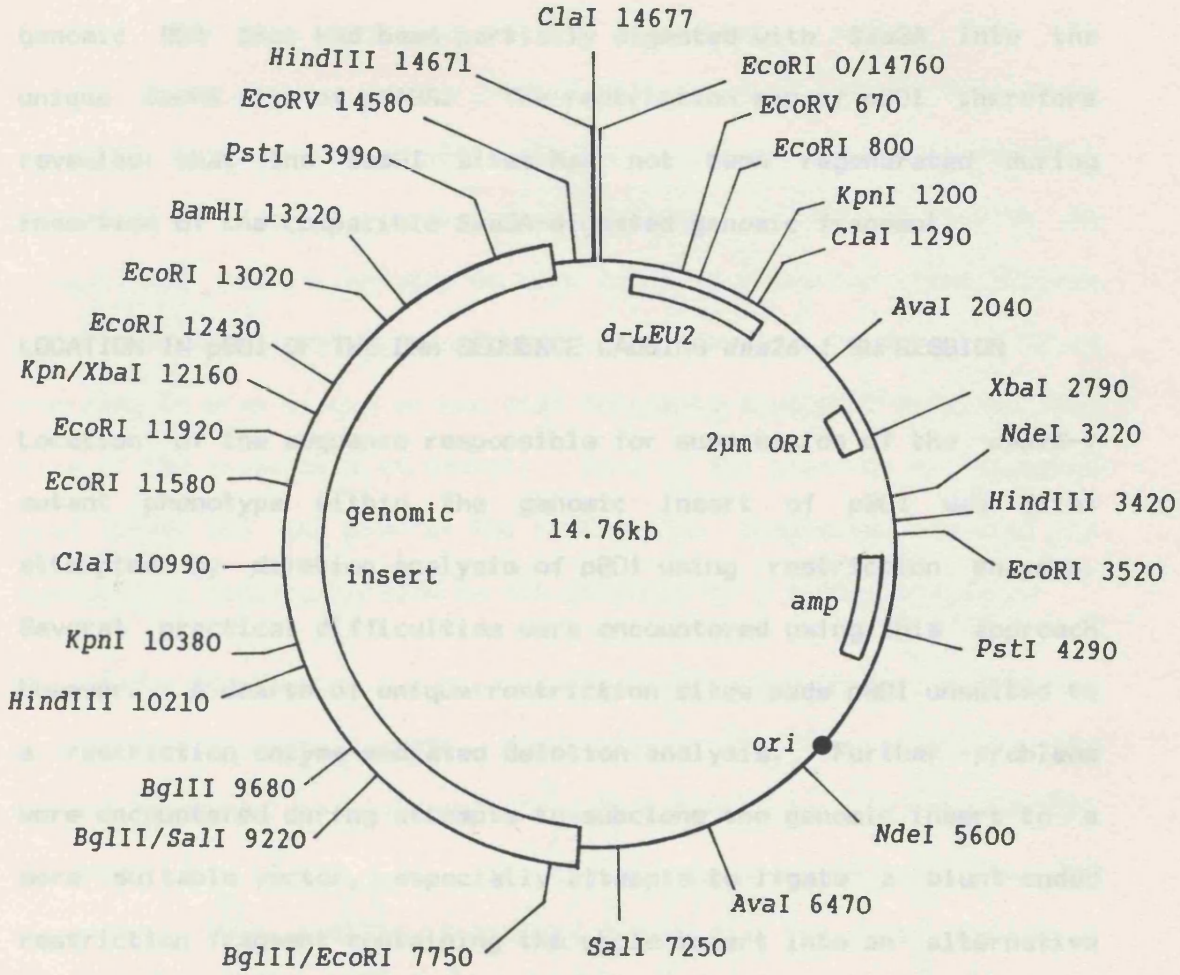


FIGURE 4.4 Restriction Map of Plasmid p801

p801 was isolated from a gene library constructed in the shuttle vector pMA3A (Appendix 1(B)). Restriction site-coordinates in the above map are approximate, based on, i) the size of fragments generated by digestion of p801 DNA, and ii) reference to the restriction maps of pMA3A and pBR322 (Appendix 1(F)). An *Eco*RI site from pMA3A has been arbitrarily designated as position zero. Cleavage sites were deduced only for those enzymes shown. The positions of the 6.86kb genomic insert and pMA3A-derived genes are shown. *d-LEU2*, defective *LEU2* gene; 2μm *ORI*, 2μm circle origin of replication; *amp*, pBR322 ampicillin resistance gene; ● *ori*, pBR322 origin of replication.

genomic DNA that had been partially digested with *Sau*3A into the unique *Bam*HI site of pMA3A. The restriction map of p801 therefore revealed that the *Bam*HI sites had not been regenerated during insertion of the compatible *Sau*3A-digested genomic fragment.

LOCATION IN p801 OF THE DNA SEQUENCE CAUSING *dna26-1* SUPRESSION

Location of the sequence responsible for suppression of the *dna26-1* mutant phenotype within the genomic insert of p801 was first attempted by deletion analysis of p801 using restriction enzymes. Several practical difficulties were encountered using this approach however. A dearth of unique restriction sites made p801 unsuited to a restriction enzyme-mediated deletion analysis. Further problems were encountered during attempts to subclone the genomic insert to a more suitable vector, especially attempts to ligate a blunt-ended restriction fragment containing the whole insert into an alternative vector. It was decided therefore to take an alternative approach of *in vivo* disruption of the cloned genomic fragment via the use of the transposon Tn5 for transposon mutagenesis of plasmid p801.

An *E. coli* strain, PCT800, was employed that contained Tn5 in its genome. The transposon had been introduced into the bacterial chromosome by infecting cells of the strain ED8767 (Murray *et al.*, 1977) with lambda phage containing a Tn5 insertion (David Dymock, personal communication). The lambda phage had been deleted for the *att* gene preventing lysogeny and mutated in the *O* and *P* genes preventing phage relication. PCT800 was transformed with the library plasmid p801 and plasmids into which Tn5 insertion had occurred were enriched for and isolated by the procedures described in section 4.2 (Materials and Methods).

Plasmid DNA was prepared by the mini-prep. method from 34 transformant colonies that displayed resistance to both kanamycin and ampicillin. Digestion of p801 with the restriction endonuclease *ClaI* generated 3 restriction fragments of 9.7kb, 3.75kb and 1.31kb in size. Tn5 itself contains no site for *ClaI* digestion (see Figure 4.5). Insertions of Tn5 into p801 could therefore be detected by an increase in size of one of the *ClaI* fragments from p801 equal to the size of the transposon (5.818kb). Each of the plasmids was digested with *ClaI* and the size of the restriction fragments measured by agarose gel electrophoresis in the presence of a lambda standard.

Out of the 34 plasmids examined, 2 displayed a Tn5 insertion into the 1.31kb *ClaI* fragment of p801, 13 displayed a Tn5 insertion into the 9.7kb fragment and 14 displayed a Tn5 insertion into the 3.75kb fragment (see Table 4.7). One plasmid appeared to contain Tn5 insertions into both the 9.7kb and 3.75kb *ClaI* fragments whilst 2 plasmids appeared not to contain a Tn5 insertion despite the kanamycin resistant phenotype of the *E. coli* transformants from which they were extracted (data not shown). Digestion of a further 2 plasmids with *ClaI* generated 4 DNA fragments in each case (data not shown). The five plasmids whose structure could not be explained by a single Tn5 insertion were omitted from further study.

The expected frequency of Tn5 insertion into the three *ClaI* fragments of p801 was calculated using the assumption that the sites of Tn5 insertion were approximately random. For p801, whose size was 14.76kb, the transposon was expected to insert once every 0.429kb during the 29 single transposition events examined. The number of insertions into the 1.31, 3.75 and 9.7kb *ClaI* fragments of p801 was therefore expected to be approximately 3, 8 and 20 respectively. The

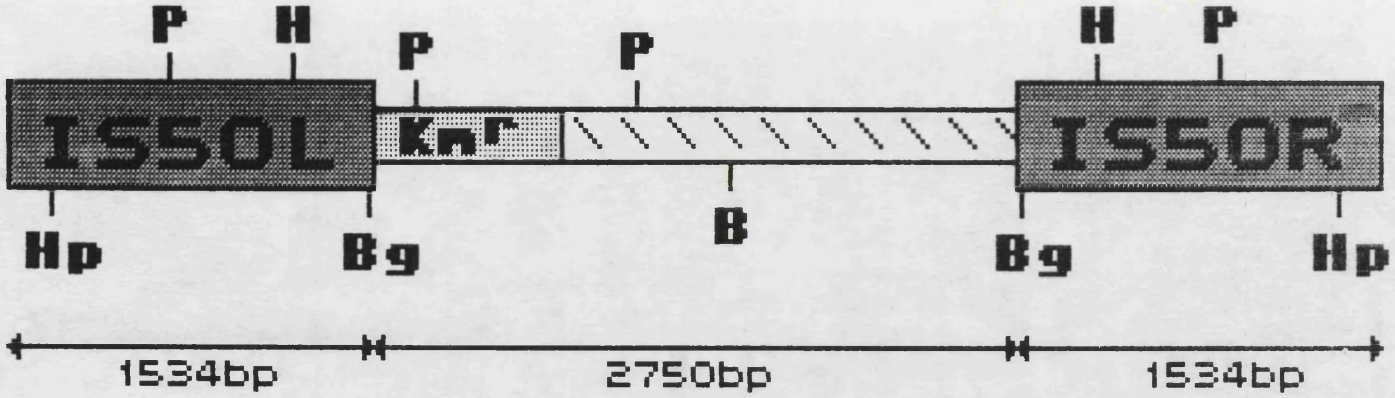


FIGURE 4.5 Physical Map of Transposon Tn5

The structure of Tn5 is shown including the size (in kb) of the two IS50 insertion sequence elements (IS50L and IS50R) and the central element carrying the kanamycin resistance marker (Kmr). The location of the various restriction sites were reproduced from data reported by Auerswald *et al.*, (1980) and Gordenin *et al.*, (1988). The distance (in bp) of the restriction sites in the IS50 elements from the outside ends are *Hpa*I(Hp), 185; *Pst*I(P), 680; *Hind*III(H), 1195; and *Bgl*II(Bg), 1515. The distances of the restriction sites in the central region of Tn5 from the outside end of IS50L are *Pst*I(P), 1728 and 2651; and *Bam*HI(B), 3056.

lower frequency of Tn5 insertion than expected into the 9.7kb *ClaI* fragment (13 insertions) could be explained by the fact that the bacterial origin of replication and ampicillin resistance marker resided in this region of the plasmid. Tn5 insertion into either *ori* or *amp*^{R} would have prevented isolation of the plasmids from the original screen. This requirement for initial selection also provided an explanation for the higher than expected frequency of Tn5 insertion into 3.75kb *ClaI* fragment of p801.

All 29 plasmids containing a single Tn5 insertion were used to separately transform the *dna26-1* mutant TDE/16A. Six transformation plates for each plasmid (consisting of selective SD medium lacking leucine) were initially incubated at 25°C for 30hr. Half of the transformation plates for each plasmid were then shifted to 36°C. The number of transformant colonies generated by each plasmid was determined after 20 days at the respective temperatures.

The results of the transformation experiments are shown in Table 4.7. The number of yeast transformants obtained with most of the plasmids was low. This was due to the fact that the transforming plasmid DNA in each case had been prepared by the mini-prep. method and had not therefore been extensively purified. For all of the plasmids that supported the growth of yeast cells at 36°C the number of transformant colonies produced at 36°C was generally slightly lower than the number produced at 25°C. The number of transformants produced at the two temperatures was however similar in all cases. It was therefore considered likely that the plasmids that generated transformants at 25°C but not at 36°C contained a Tn5 insertion into the plasmid-borne gene responsible for suppression of the *dna26-1* mutant phenotype. A total of 8 plasmids, namely pTn5-N, -H, -C, -T,

PLASMID (pTn5-)	NUMBER OF TRANSFORMANTS 25°C	36°C	*Cla*I FRAGMENT (kb)	PLASMID (pTn5-)	NUMBER OF TRANSFORMANTS 25°C	36°C	*Cla*I FRAGMENT (kb)
51	1	3	1.31	66	0	0	9.7
67	110	105	1.31	70	0	0	9.7
G	16	5	3.75	A	3	4	9.7
L	5	8	3.75	B	25	16	9.7
Q	29	12	3.75	E	22	18	9.7
52	15	10	3.75	I	33	19	9.7
54	3	2	3.75	J	5	12	9.7
C	32	0	3.75	K	6	7	9.7
DN	0	0	3.75	M	31	24	9.7
H	16	0	3.75	P	13	6	9.7
N	2	0	3.75	S	8	9	9.7
R	5	0	3.75	53	74	42	9.7
T	5	0	3.75	71	3	2	9.7
55	10	0	3.75	p801*	7	7	---
57	102	0	3.75	pMA3A*	4	0	---
63	21	0	3.75	- DNA**	0	0	---

TABLE 4.7 Ability of p801-Tn5 Plasmids to Transform TDE/16A

The data are for the number of Leu$^+$ transformant colonies produced on transformation plates that were incubated at 25°C or 36°C (see text). The *Cla*I fragment of p801 into which Tn5 insertion had occurred is also shown.

* p801 and pMA3A were included as positive and negative controls for colony formation at 36°C respectively.

** Competent cells plated in the absence of plasmid DNA.

-R, -55, -63 and -57, successfully generated Leu$^+$ transformants at 25°C but failed to do so at 36°C. All of these plasmids contained a Tn5 insertion into the 3.75kb *ClaI* fragment of p801. A further 5 plasmids containing a Tn5 insertion into the 3.75kb *ClaI* fragment produced transformant colonies at both 25°C and 36°C. All 11 plasmids containing a Tn5 insertion into the 9.7kb *ClaI* fragment of p801 and that generated Leu$^+$ transformants at 25°C also generated a similar number of transformants at 36°C. The other 2 plasmids containing a Tn5 insertion into the 9.7kb *ClaI* fragment each failed to produce transformant colonies. A certain number of such plasmids were expected because part of the *d-LEU2* gene required for plasmid selection and all of the *2μm* origin of replication and *REP3* sequences required for plasmid replication were situated in this 9.7kb region of p801. Finally, the 2 plasmids that contained a Tn5 insertion into the 1.31kb *ClaI* fragment of p801 both generated Leu$^+$ transformants of TDE/16A at both 25°C and 36°C. This suggested that the Tn5 insertions into these plasmids had not disrupted the *d-LEU2* gene present in this region of p801.

The above data strongly suggested that the gene responsible for suppression of the *dna26-1* mutation was located within the 3.75kb *ClaI* fragment of p801. A second series of transformations was therefore carried out for those plasmids that contained a Tn5 insertion into the 3.75kb *ClaI* fragment and that had failed to generate ts$^+$ transformants in the initial transformations. Larger quantities of plasmid DNA prepared by the midi-prep. method were used to increase the number of transformants obtained, at least at 25°C. Similar results were obtained in this experiment as in the first (Table 4.8). All of the plasmids examined transformed TDE/16A to

PLASMID (pTn5-)	NUMBER OF TRANSFORMANTS 25°C	36°C
DN	>800	0
H	>200	0
N	>1000	20
R	34	0
T	72	1
54	>800	>800
55	26	0
p801*	200	154
pMA3A**	4	0
- DNA#	0	0

TABLE 4.8 The Ability of p801-Tn5 Plasmids to Transform TDE/16A

The data are for the number of Leu$^+$ transformants produced at 25°C and 36°C by the relevant plasmids. Transformation plates (10X SD -leucine plates per plasmid) were initially incubated at 25°C for 30hr. Half of the plates for each plasmid were then shifted to 36°C. Transformant colonies were counted after 20 days at the respective temperatures. All the pTn5- plasmids contained an insertion of transposon Tn5 into the 3.75kb *ClaI* fragment of plasmid p801 and were prepared by the midi-prep. method. * p801 was included as a positive control for transformation of TDE/16A to a ts$^+$ phenotype. ** pMA3A was included as a negative control for transformation of TDE/16A to a ts$^+$ phenotype. # competent cells were treated with TE buffer containing no DNA, spread onto selective SD plates and incubated at 25°C or 36°C.

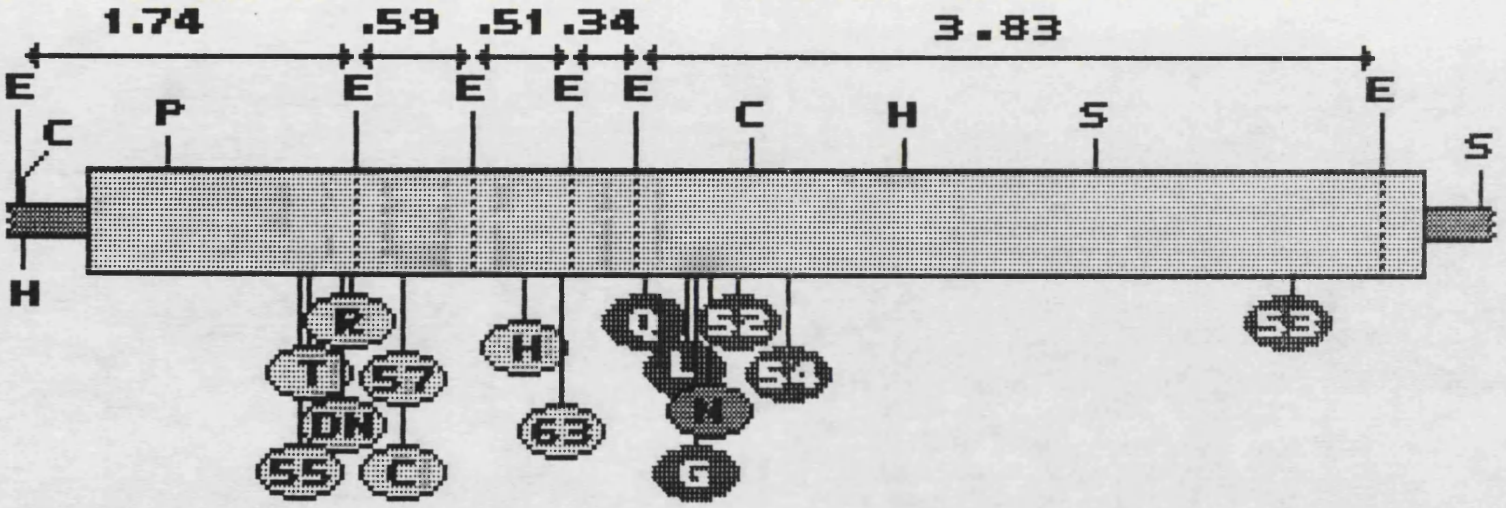
leucine independence at high frequency when transformants were selected at 25°C, whilst no Leu$^+$ transformants were obtained at 36°C. One further plasmid (pTn5-DN), containing a Tn5 insertion into the 3.75kb *Cla*I fragment of p801, was also shown by this second transformation experiment to be defective in the suppression of the *dna26-1* mutation at 36°C. In addition, the ability of pTn5-54 to generate ts$^+$ transformants was confirmed by this experiment.

In order to locate the precise region within p801 that contained the *dna26-1*-suppressing activity, further restriction analysis was carried out on 15 of the plasmids containing an insertion of Tn5 into or near to the 3.75kb *Cla*I fragment. Each plasmid was first digested with *Eco*RI for which there were several closely spaced cleavage sites neighbouring the central *Cla*I site in the genomic insert of p801. Tn5 itself contains no *Eco*RI sites. Digestion of the mutagenized plasmids with *Eco*RI therefore permitted the localization of Tn5 insertions to one of the *Eco*RI fragments from p801 (see Figure 4.6).

Finally, in an attempt to fine-map the Tn5 insertions to an approximate base pair coordinate within p801, the 15 Tn5-containing plasmids were digested with the *Hind*III restriction endonuclease. Plasmid p801 had three sites for digestion by *Hind*III whilst Tn5 has two sites which are 1195bp from the outer ends of IS50L and IS50R. Digestion of p801 containing one copy of Tn5 therefore generated five restriction fragments. Two of these fragments corresponded to two of the intact p801 *Hind*III fragments whilst one corresponded to the 3.428kb central *Hind*III fragment of Tn5. The other two fragments generated each contained a portion of one of the IS50 elements (totalling 1195bp) with the rest of the fragment consisting of

FIGURE 4.6 The location of Tn5 Insertions into the Genomic Insert in Plasmid p801

Fifteen separate Tn5 insertions into the cloned fragment in p801 are shown (lower portion of map). The restriction sites for *Eco*RI (E), *Hind*III(H), *Cla*I(C), *Pst*I(P) and *Sal*I(S) in the genomic insert are also shown (upper portion of map). The separation of the *Eco*RI sites (in kb) are indicated by the headed arrows. The exact location of each Tn5 insertion was calculated as a coordinate within p801 by digestion with *Hind*III. The coordinates of those Tn5 insertions that disrupt *dna26-1* suppression are 55(13.27), T(13.26), R(13.03), H(12.15), DN(13.10), 57 and C(12.78) and 63(11.96). The coordinates of the insertions that do not disrupt *dna26-1* suppression are Q(11.52), G(11.33), L(11.32), 52(11.07), 54(10.80) and 53(8.195). The Tn5 insertion into pTn5-N (coordinate 11.20) partially disrupts *dna26-1* suppression (see text). The bounderies of the cloned insert are located at coordinates 14.39 and 7.53b.



6.8kb GENOMIC INSERT IN PLASMID p801

PORTION OF p801 VECTOR SEQUENCES

Tn5 INSERTIONS THAT DISRUPT SUPPRESSION OF *dna26-1*

Tn5 INSERTIONS THAT DO NOT DISRUPT SUPPRESSION OF *dna26-1*

pTn5-N DISPLAYS PARTIAL SUPPRESSION OF *dna26-1* (see text)

sequences derived from one of the *Hind*III fragments from p801. The generation of these two latter fragments created an ambiguity in the location of a Tn5 insertion as judged by the *Hind*III data alone. Mapping by *Hind*III digestion was therefore carried out by firstly predicting the approximate position of a Tn5 insertion from the *Cla*I and *Eco*RI restriction data and secondly fine mapping the point of insertion using the *Hind*III data (see legend to Figure 4.6).

Mapping of the Tn5 insertions in plasmids pTn5-55 and pTn5-63 at coordinates 13.28 and 11.96 respectively established the lower limit for the size of the *dna26-1*-suppressing sequence in p801 as 1.35kb (see Figure 4.6). The outer limit for the size of the suppressor was set at 2.88kb by the Tn5 insertion into pTn5-Q at coordinate 11.52 (which did not disrupt suppressor activity) and the plasmid/genomic insert junction at coordinate 14.39. These size estimates relied upon the assumption that the suppressor contained no introns and were indicative of a medium sized *S. cerevisiae* gene (Mosse *et al.*, 1988).

Only two anomalous results arose from the Tn5 data. Firstly the Tn5 insertion in pTn5-N at coordinate 11.20 appeared to partially disrupt suppression of the *dna26-1* mutant phenotype (see Table 4.8). This was unexpected as four adjacent Tn5 insertions at coordinates 11.52(Q), 11.32(L), 11.33(G) and 11.07(52) respectively did not disrupt *dna26-1* suppression. It is possible that the insertion in pTn5-N interfered with the regulation of expression of the suppressor in a way that the insertions in pTn5-Q, -L -55 and -G did not. The second anomalous result concerned the site of Tn5 insertion in pTn5-54. The *Hind*III restriction data mapped this insertion to coordinate 10.80 which lay within the 9.7kb *Cla*I fragment of p801. The *Cla*I data on the other hand mapped this insertion to the 3.75kb *Cla*I

fragment. This anomaly almost certainly derived from an inaccuracy in the electrophoretic analysis of the *Hind*III fragments that were generated from pTn5-54. The Tn5 insertion in this plasmid was probably actually situated at a coordinate in p801 greater than 10.99 (the coordinate of the *Cla*I site in the genomic insert).

SUB-CLONING OF THE SUPPRESSOR SEQUENCE TO A LOW COPY VECTOR

The plasmid library from which p801 was isolated was based on the vector pMA3A. pMA3A has a copy number of 100-150 copies per cell in yeast (Spalding & Tuite, 1989). This is due to the presence in pMA3A of the origin of replication, *REP3* gene, inverted repeat and *RAF* sequences derived from the endogenous *S. cerevisiae* 2*µm* plasmid. pMA3A also contains the *d-LEU2* marker which contains a deletion for its promoter sequences and must be expressed at high copy number to complement a *leu2* mutation (Erhart & Hollenberg, 1983). It was therefore considered possible that the cloned sequence in p801 might contain a suppressor gene that was only capable of functionally complementing the *dna26-1* mutation at high copy number. Furthermore, because p801 contained a large (6.8kb) insert, it was thought probable that the insert contained more than one gene and possible that the suppression of the *dna26-1* mutation observed may have been due to the interactive function of closely linked genes.

It was therefore decided to sub-clone the 3.75kb *Cla*I fragment from p801 into the low copy vector YCp50. YCp50 replicates at a copy number of 1 or 2 per cell in yeast due to the presence of centromeric sequences from chromosome IV (Rose *et al.*, 1987). YCp50 also contains several unique restriction sites for the endonucleases that were employed in the mapping of p801 making further deletion

analysis of the 3kb *ClaI* fragment a possibility if necessary. It was also decided to sub-clone the 3kb *ClaI* fragment into the multi-copy vector YEp24 (Botstein *et al.*, 1979; Appendix 1(D)). This was carried out to provide verification of the Tn5 data for the location of the *dna26-1*-suppressing activity within the cloned fragment. Furthermore, the sub-cloning of the 3kb fragment to both a high copy and a low copy plasmid (YEp24 and YCp50 respectively) provided a means to establish whether it contained the wild type *DNA26* gene or a dosage suppressor.

Both YCp50 and YEp24 contain a unique *ClaI* site and can therefore be linearized by digestion with the *ClaI* restriction enzyme. The 3kb *ClaI* fragment from p801 was isolated by digestion of the plasmid with *ClaI* followed by agarose gel electrophoretic separation of the three restriction fragments generated. The 3kb *ClaI* fragment was then separately ligated into the *ClaI* site of both YEp24 and YCp50. The ligation mixtures were used to transform *E. coli* strain DH5alpha to ampicillin resistance. 750 and 150 transformants were obtained for the ligation mixtures containing YCp50 and YEp24 respectively.

The plasmid DNA was prepared from 12 ampicillin resistant *E. coli* transformants for each plasmid source. The successful insertion of the cloned fragment into both YEp24 and YCp50 and its orientation in these plasmids was determined by digestion with the restriction endonuclease *HindIII* and analysis of the restriction fragments by agarose gel electrophoresis. 6 out of 12 of the YEp24 plasmids analyzed contained a single copy of the cloned 3kb *ClaI* fragment; 4 with the cloned sequence in one orientation and 2 with it in the other orientation (Plate 4.3). The structures of two of the YEp24 plasmids with the cloned sequence inserted in the alternative

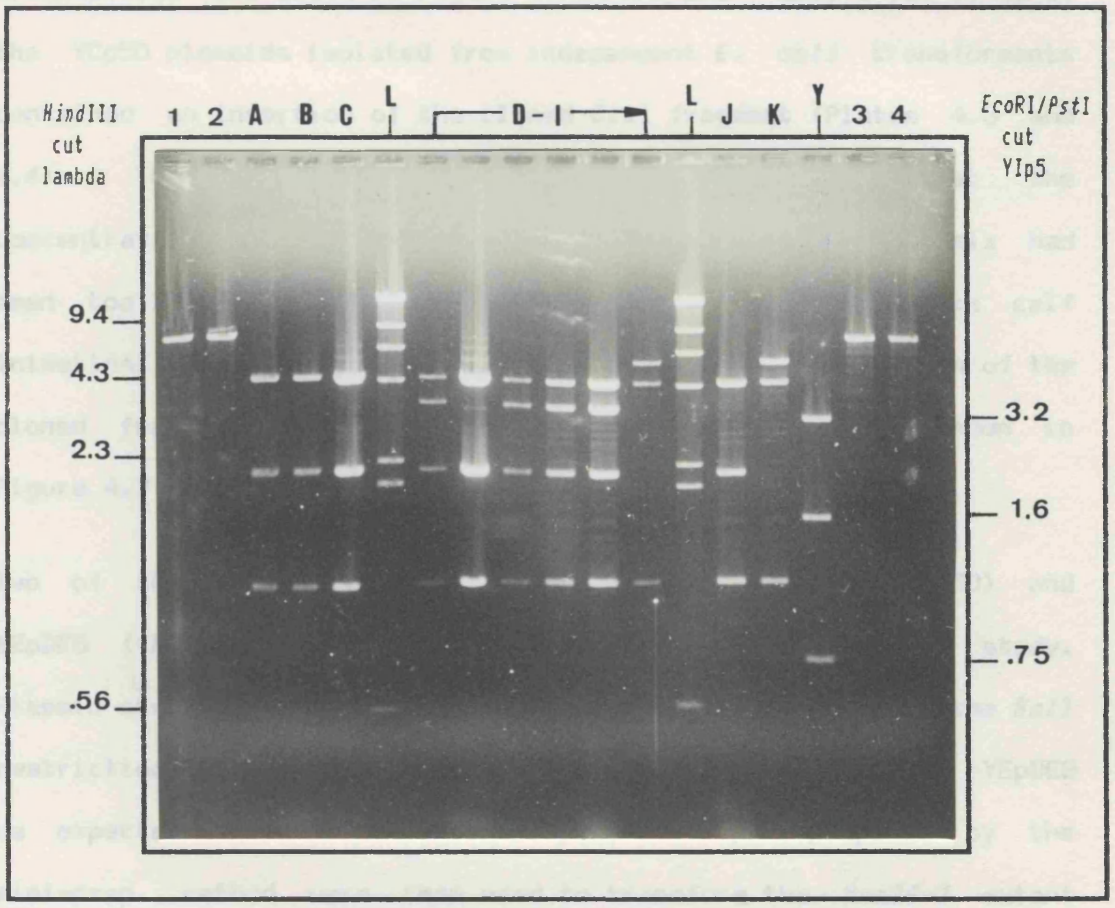


PLATE 4.3 Restriction Analysis of Constructs Made in YEp24 and YCp50

Plasmid constructs generated from ligation reactions containing the 3kb *Cla*I fragment from p801 and either YEp24 or YCp50 were digested with *Hind*III and subjected to agarose gel electrophoresis. Lanes: 1-4, Ycp50-based constructs; A-K, YEp24-based constructs; L, lambda(*Hind*III); Y, YIp5(*Eco*RI/*Pst*I). Single insertions into YEp24 in the two alternative orientations are represented in i) lanes D, F, G, and H, and ii) lanes I and K, respectively (see Figure 4.10). None of the above YCp50 plasmids contain insertions.

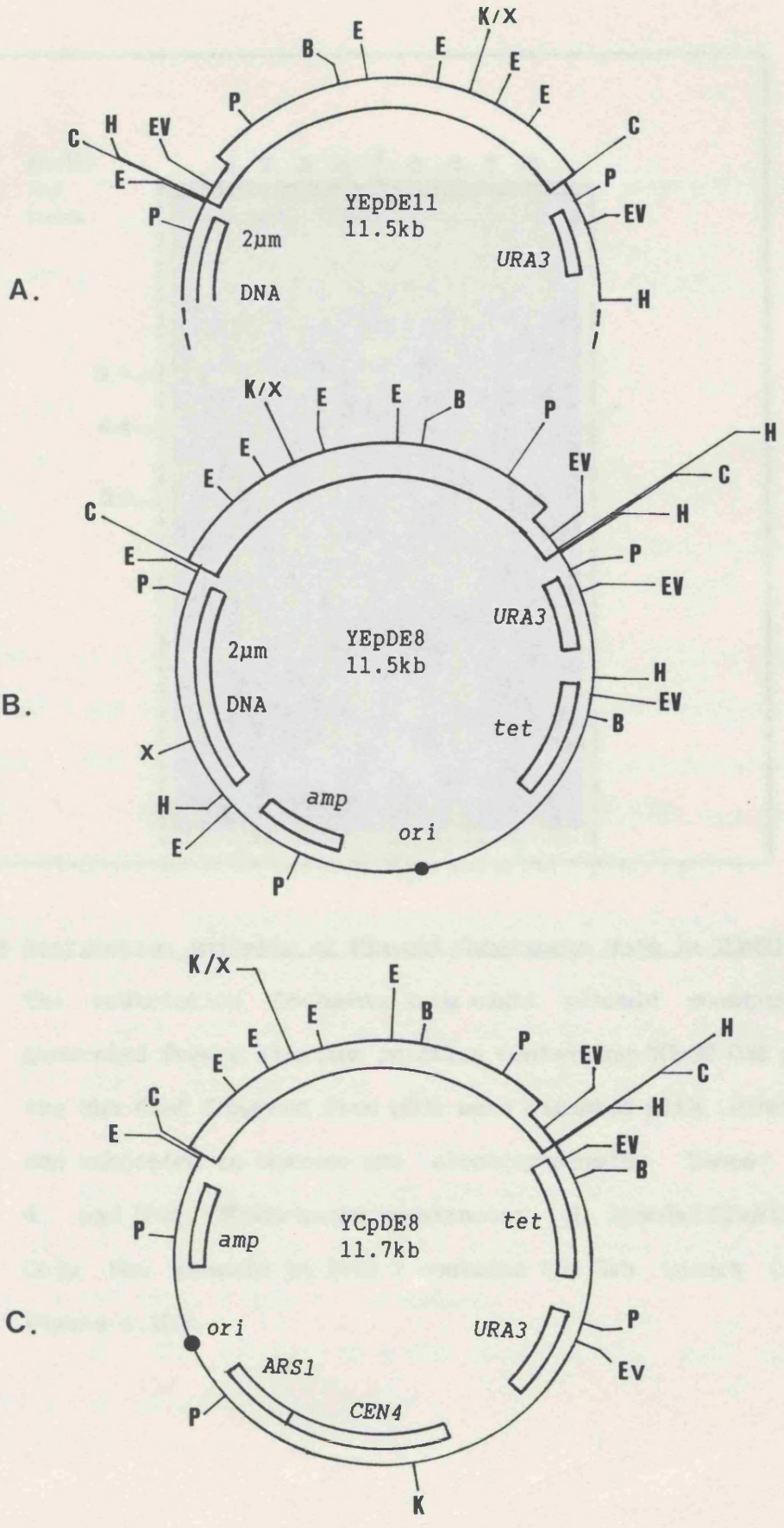
orientations are shown in Figure 4.7(A and B). Only 1 out of 12 of the YCp50 plasmids isolated from independent *E. coli* transformants contained an insertion of the cloned *ClaI* fragment (Plates 4.3 and 4.4). This low frequency of insertion suggested that the concentration of the 3kb cloned fragment in the ligation mix had been too low and that the dephosphorylation of vector DNA with calf intestinal phosphatase had been inefficient. The orientation of the cloned fragment in the one recombinant plasmid obtained is shown in Figure 4.7(C).

Two of the plasmid constructs YCpDE8 (the 3kb clone in YCp50) and YEpDE8 (the 3kb clone in YEp24) were selected for further study. Plasmid structure was rechecked by restriction analysis with the *SalI* restriction endonuclease which linearized both YCpDE8 and YEpDE8 as expected (data not shown). YCpDE8 and YEpDE8 prepared by the mini-prep. method were then used to transform the *dna26-1* mutant TDE/16A. Both plasmids contained the *URA3* marker permitting transformant colonies to be selected on SD medium lacking uracil. Half of the transformation plates for each plasmid were shifted from 25°C to 36°C after 48hr and the number of transformant colonies produced at the respective temperatures scored after 10 days. Both YCpDE8 and YEpDE8 DNA were capable of transforming TDE/16A to a ts^+ phenotype (Table 4.9).

The ability of YCpDE8 to suppress the *dna26-1* temperature sensitive mutant phenotype confirmed the Tn5 mutagenesis data for the location of the suppressor sequence in the 3kb *ClaI* fragment from p801. It further suggested that the wild type *DNA26* gene rather than a dosage suppressor of the *dna26-1* mutation had been cloned. In order to confirm this it was decided to examine the degree of linkage between

FIGURE 4.7 Orientation of Insertions into YEp24 and YCp50

The figure shows the orientation of the 3.75kb *ClaI* fragment from p801 inserted into YEp24 and YCp50 (see Appendices 1(D) and 1(A) respectively). YEpDE11 (A) and YEpDE8 (B) were obtained by ligation of the 3.75kb *ClaI* fragment into YEp24 (see lanes H and K respectively in Plate 4.3). Only a portion of YEpDE11 is shown. YCpDE8 (C) was obtained by ligation of the same *ClaI* fragment into YCp50 (see lane 7, Plate 4.4). In each map the locations of the vector-derived genes are shown as inset boxes within the circumference of the molecule. The outer box represents the 3.75kb *ClaI* fragment from p801 which contains yeast (full box-width) and pBR322 (half box-width) sequences. Yeast vector DNA - *URA3*, wild type gene encoding orotidine-5'-phosphate carboxy-lyase; 2*µm* DNA, 2.2kb *Eco*RI fragment from 2*µm* circle (B-Form); *ARS1*, autonomous replication sequence; *CEN4*, centromeric sequences from chromosome IV. pBR322 DNA - *amp* and *tet*, ampicillin and tetracycline resistance genes respectively; *ori*, origin of replication. Restriction sites - E, *Eco*RI; H, *Hind*III; C, *Cla*I; P, *Pst*I; EV, *Eco*RV; B, *Bam*HI; X, *Xba*I; K, *Kpn*; K/X, closely adjacent *Kpn*I and *Xba*I sites. Only the sites for the latter endonucleases are shown.


A.
YEpDE11
11.5kb
2µm
DNA
URA3
K/X
E
B
P
EV
C
H
B.
YEpDE8
11.5kb
2µm
DNA
URA3
tet
amp
ori
X
C.
YCpDE8
11.7kb
tet
amp
ori
URA3
ARS1
CEN4
Ev
K

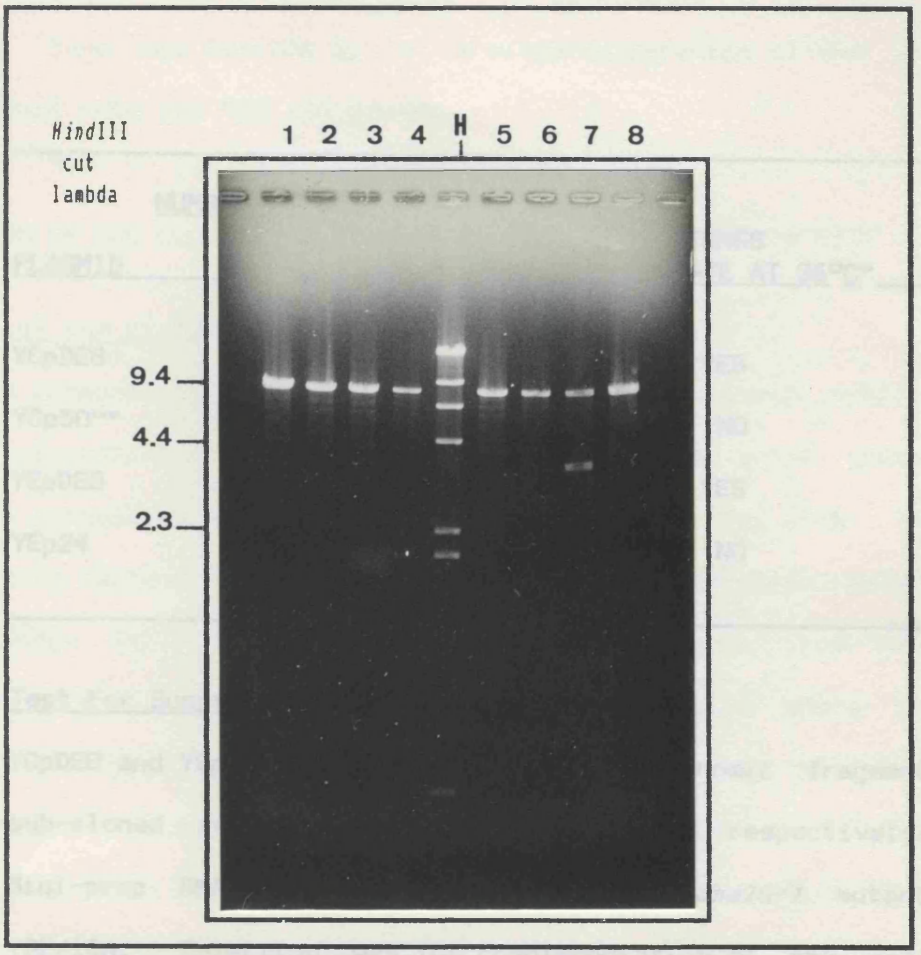


PLATE 4.4 Restriction Analysis of Plasmid Constructs Made in YCp50

The restriction fragments from eight plasmid constructs generated from a ligation reaction containing YCp50 DNA and the 3kb *Cla*I fragment from p801 were digested with *Hind*III and subjected to agarose gel electrophoresis. Lanes: 1-4, and 5-8, YCp50-based constructs; H, lambda(*Hind*III). Only the plasmid in lane 7 contains the 3kb insert (see Figure 4.10).

PLASMID	NUMBER OF TRANSFORMANTS 25°C	36°C	SUB-CULTURES PROLIFERATE AT 36°C*
YCpDE8	9	4	YES
YCp50**	65	0	NO
YEpDE8	9	3	YES
YEp24	4	0	NO

TABLE 4.9 Test For Suppression of the *dna26-1* Mutation

YCpDE8 and YEpDE8 contain the 3.75kb *ClaI* genomic fragment sub-cloned from p801 into YCp50 and YEp24 respectively. Mini-prep DNA was used to transform the *dna26-1* mutant TDE/16A. Selection was for complementation of the *ura3* mutation in TDE/16A by the plasmid borne *URA3* marker in each case. Half of the transformation plates for each plasmid were shifted from 25°C to 36°C after 48hr. Transformant colonies were counted after 10 days of incubation.

* Single transformant colonies were transferred from 25°C to 36°C by streaking for single colonies. Colony formation was scored after 4 days of incubation.

** YCp50 DNA purified on a CsCl gradient was used to transform TDE/16A.

the *dna26-1* mutation and the genomic locus homologous to the cloned fragment. This was carried out by directed integration of the 3kb *Cla*I fragment into the TDE/16A genome.

INTEGRATION OF THE CLONED SEQUENCE AT ITS HOMOLOGOUS GENOMIC SITE

Insertion of the cloned 3kb *Cla*I Fragment into YIp5

The yeast integrating vector YIp5 was chosen as a suitable vehicle for the integration of the 3kb *Cla*I fragment into the yeast genome due to its possession of the *URA3* marker and a single *Cla*I site. The plasmid also lacks a cleavage site for the *Xba*I restriction enzyme (see Appendix 1(E)). The cloned 3kb *Cla*I fragment from p801 contained a single *Xba*I site located in the region where Tn5 insertion had been shown to disrupt *dna26-1*-suppressing activity. The proposed strategy was therefore to linearize the integration vector within the sub-cloned 3kb *Cla*I sequences by digestion with *Xba*I and thereby direct integration of the clone to its homologous site in the TDE/16A genome during transformation and selection for uracil independence (Orr-Weaver *et al.*, 1981).

The 3kb *Cla*I fragment was separated from the 9kb and 1kb *Cla*I fragments of p801 by gel electrophoresis and added to a ligation reaction mixture containing dephosphorylated YIp5 DNA that had been linearized by digestion with *Cla*I. Due to initial problems with ligation reactions a further ligation mixture was prepared containing linearized YIp5 DNA and all three *Cla*I fragments from p801 obtained from the restriction digest without gel separation. The ligation mixtures were used to transform *E. coli* strain DH5alpha to ampicillin resistance.

22 ampicillin resistant transformants were obtained from the ligation mixture containing the gel-separated cloned fragment whilst 57 transformants were obtained from the ligation mixture containing all the *Cla*I digestion products from p801. Plasmid DNA from randomly selected transformants was prepared by the mini-prep. method and structurally analysed by restriction analysis with *Hind*III. The *Hind*III sites in the two larger *Cla*I fragments from p801 and in the YIp5 vector DNA permitted the identification of all ligation products.

The ligation reaction containing the mixture of *Cla*I fragments from p801 yielded a number of different plasmid constructs whose structure could be determined by the *Hind*III restriction analysis (Plate 4.5). However, out of the 8 plasmids analysed none contained an insertion of the 3kb *Cla*I fragment alone. From the ligation reaction containing the purified 3kb *Cla*I fragment, 6 out the 10 plasmids analysed contained a 3kb insert (Plate 4.5). All 6 plasmid constructs surprisingly contained the insert in the same orientation. The structure of one of these plasmids, designated YIpDE8, was further checked by digestion with the restriction enzymes *Cla*I, *Pst*I and *Xba*I (data not shown). The structure of YIpDE8 is shown in Figure 4.8.

Directed Integration of the Cloned Sequence

YIpDE8 DNA was prepared by the midi-prep. method. The plasmid was linearized at the unique *Xba*I site within the cloned insert by restriction digestion. Approximately 5μg of the linear plasmid DNA were used to transform the *dna26-1* mutant TDE/16A. 6 transformant colonies were initially selected on SD medium lacking uracil at 25°C.

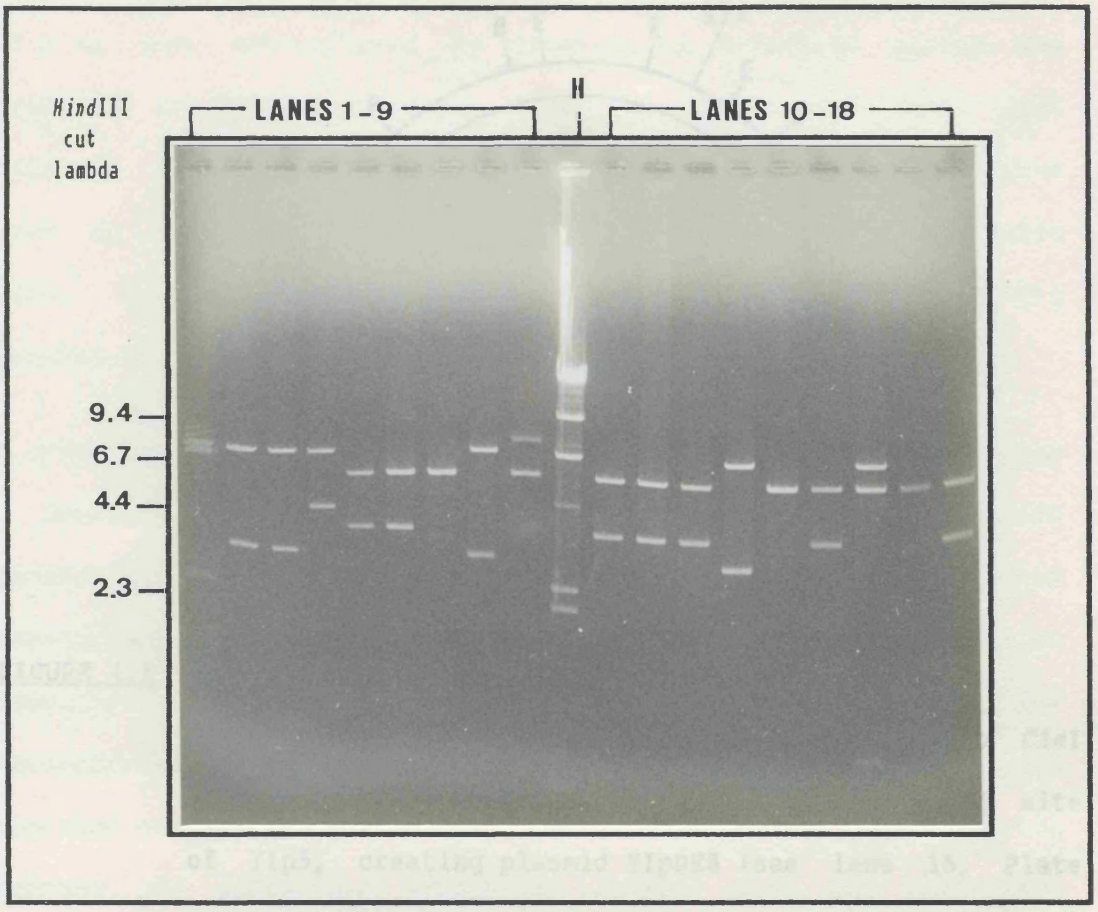


PLATE 4.5 Restriction of Analysis of Constructs Made in YIp5

Eighteen plasmid constructs generated from a ligation reaction containing YIp5(*Cla*I) DNA and the 3kb *Cla*I fragment from p801 were digested with *Hind*III and subjected to agarose gel electrophoresis. Lanes: 1-9 and 10-18, YIp5-based constructs; H, lambda(*Hind*III). Constructs in lanes 5, 6, 10, 11, 12, 15 and 18 consist of a single insertion of the cloned fragment into YIp5.

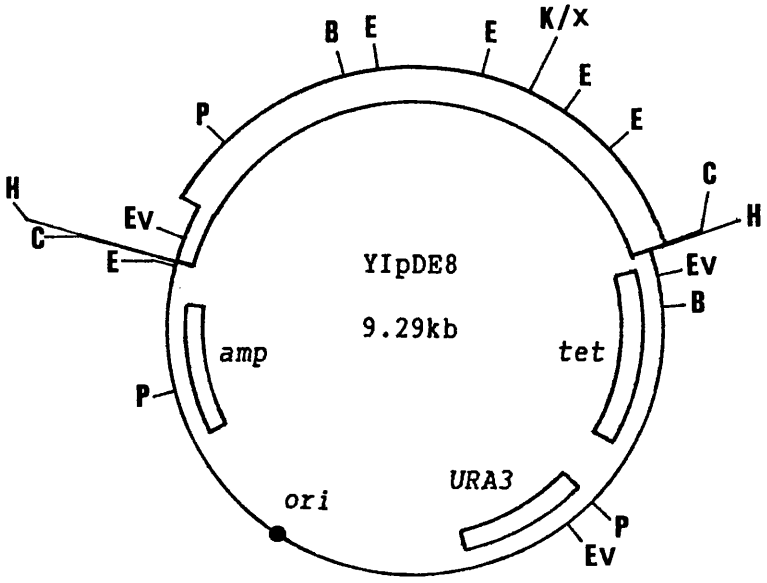


FIGURE 4.8 Orientation of the Fragment Ligated into YIp5

The figure shows the orientation of the 3.75kb *Cla*I fragment from p801 after insertion into the *Cla*I site of YIp5, creating plasmid YIpDE8 (see lane 15, Plate 4.8). Vector-derived genes are shown as inset boxes within the circumference of the map (see Appendix 1(E) for YIp5 map). The outer box represents the 3kb *Cla*I fragment from p801 which contains yeast (full box-width) and pBR322 sequences (half box-width). *URA3*, wild type yeast marker; *amp* and *tet*, ampicillin and tetracycline resistance genes respectively; *ori*, pBR322 origin of replication. E, *Eco*RI; H, *Hind*III; C, *Cla*I; P, *Pst*I; Ev, *Eco*RV; B, *Bam*HI; X, *Xba*I; K, *Kpn*I; K/X, closely adjacent *Kpn*I and *Xba*I sites. Only the sites for the latter endonucleases are shown.

These transformants were able to proliferate and produce single colonies when sub-cultured by streaking to selective medium and incubated at 36°C for 4 days. Each of the transformants also displayed a very high stability of the Ura^+ and ts^+ phenotypes when grown on non-selective liquid medium to high cell density (Table 4.10). Each transformant therefore displayed the characteristics expected for an integration event involving YIpDE8.

In order to confirm the integration of YIpDE8 into the TDE/16A genome a Southern analysis of genomic DNA from one of the stable transformants was carried out. Genomic DNA (10μg) from the transformant strain IT3 was digested with the *BglII* restriction endonuclease and the resulting DNA fragments separated by agarose gel electophoresis. Similarly digested genomic DNA from TDE/16A was separated on the same gel. The DNA was then transferred to a nylon membrane and probed with YIpDE8 DNA labelled with the nucleotide analogue digoxigenin-11-dUTP.

The YIpDE8 probe detected 2 *BglII* fragments in the genomic DNA from both the untransformed mutant TDE/16A and the integrant strain IT3 (Plates 4.6A and B). A fragment of approximately 4.6kb in size was detected in the genomic DNA from both strains. The *URA3* sequences in YIpDE8 were not removed prior its use as a probe and it was assumed that the 4.6kb fragments corresponded to a genomic *BglII* fragment containing the *URA3* gene. This assumption relied upon evidence from a previously published chromosomal restriction map of a region of chromosome V encompassing the *URA3* locus (Rose *et al.*, 1984). This map reveals the *URA3* locus to be present on a 4kb genomic *BglII* fragment.

STRAIN	GROWTH CONDITIONS +uracil/36°C	-uracil/25°C	+uracil/25°C	PERCENTAGE OF PLASMID LOSS*
IT1	98	97	100	2
IT2	100	99	100	0
IT3	100	100	100	0
IT4	100	100	100	0
IT5	98	100	100	0
IT6	100	100	100	0
TDE/16A** (YEpDE8)	82	82	100	18

FIGURE 4.10 Number of Viable Colonies After Growth of Transformants Under Non-Selective Conditions

The phenotypic stability of strains resulting from the transformation of TDE/16A by linearized YIpDE8 was analysed after growth on non-selective medium. Culture samples were diluted, spread onto non-selective plates and incubated at 25°C for 3 days. The colonies were then replica-plated to the growth conditions shown in the table and colony formation was scored after 4 days of incubation at the relevant temperatures.

* Scored on the criterion of loss of both the ts$^+$ and Ura$^+$ phenotypes that are otherwise expected to be conferred by the presence of YIpDE8.

** A transformant of TDE/16A containing the autonomously replicating vector YEpDE8 carrying the cloned 3kb *ClaI* fragment was included as a control showing a mitotically unstable transformed phenotype.

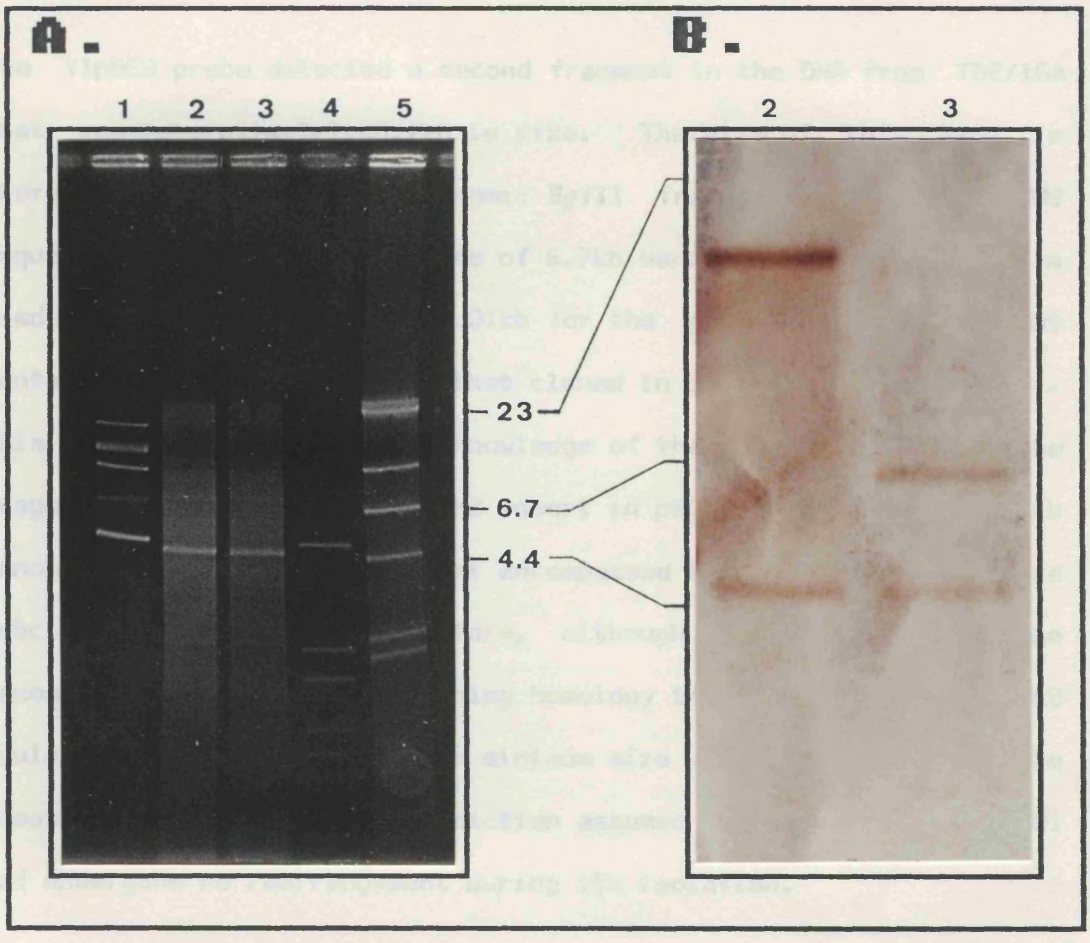


PLATE 4.6 Southern Analysis of the Genomic DNA of Transformant IT3

20µg of genomic DNA from TDE/16A and the transformed derivative IT3 was A, digested with *Bgl*II and separated by agarose gel electrophoresis, and B, transferred to a nylon membrane and probed with digoxigenin-labelled YIpDE8 DNA (only lanes 2 and 3 shown).

1. Linearized p801(14.8kb), pBR328(4.9kb) and YIpDE8(9.3kb) - (partial restriction fragments also present).
2. Genomic DNA of integrant strain IT3 digested with *Bgl*II.
3. Genomic DNA of TDE/16A digestesd with *Bgl*II.
4. pBR328 separately digested with *Eco*RI, *Bgl*I and *Hinf*I.
5. lambda(*Hind*III).

The YIpDE8 probe detected a second fragment in the DNA from TDE/16A that was approximately 6.7kb in size. The size of this band is therefore larger than the genomic *BglII* fragment containing *URA3* sequences. Moreover, the size of 6.7kb band was consistent with a predicted minimum size of 4.31kb for the genomic *BglII* fragment containing DNA homologous to that cloned in p801 (see Figure 4.9). This prediction was made from knowledge of the restriction map of the fragment cloned in p801. The insert in p801 contained a 4.31kb genomic *BglII-PstI* fragment that encompassed the sequences that were subcloned into YIp5. Therefore, although the exact size of the genomic *BglII* fragment containing homology to the insert in YIpDE8 could not be predicted, its minimum size was anticipated to be greater than 4.31kb. This prediction assumed that the insert in p801 had undergone no rearrangement during its isolation.

The genomic DNA of the stable transformant IT3 apparently lacked the 6.7kb *BglII* fragment observed in the DNA from TDE/16A. The probe instead detected a higher molecular weight band estimated to be between 15kb and 20kb in size. The size of this band was consistent with the integration of a single copy of YIpDE8 at the genomic site containing homology to its insert (see Figure 4.9). The YIpDE8 construct contained no sites for *BglII* digestion. Integration of YIpDE8 into the genomic *BglII* fragment containing insert homology was therefore predicted to increase the size of that genomic fragment by 9.29kb (the size of YIpDE8). As the minimum size of the genomic fragment prior to the integration event was predicted to be 4.31kb, the minimum size of the corresponding fragment subsequent to the integration of a single copy of YIpDE8 was predicted to be 13.6kb. Furthermore, from the size of the corresponding "pre-integration"

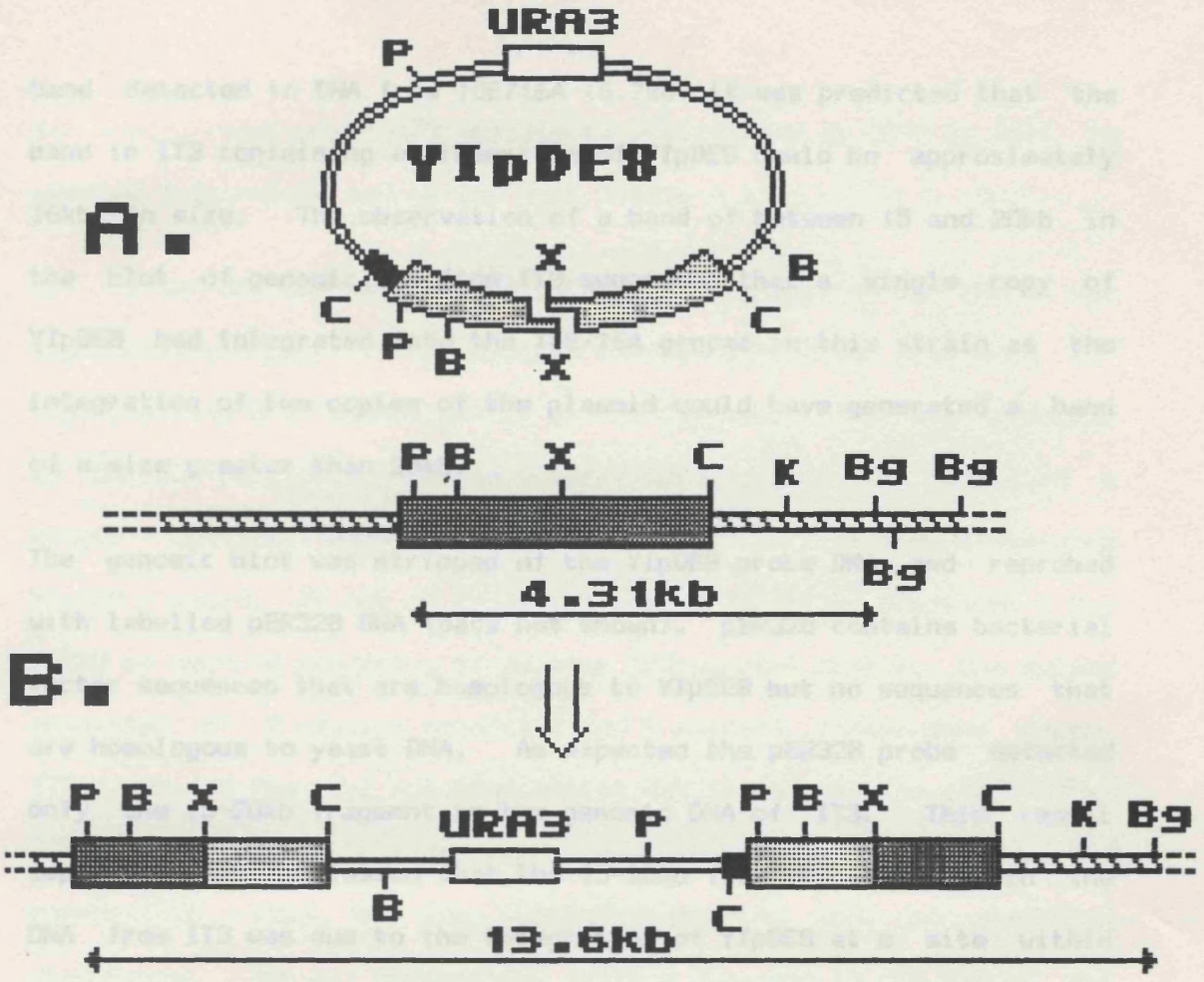
Figure 4.9

Model for the Integration of a Single copy of YIpDE8(*XbaI*)

A. Directed integration of YIpDE8 (9.29kb) by homologous recombination with the TDE/16A(*dna26-1*) genome after linearization of the plasmid within the 3kb cloned fragment. Prior to integration, the genomic *BglII* fragment that contains sequences that are homologous to the 3kb clone is of uncertain size, but at least 4.31kb (see p801 map).

B. The structure of genomic DNA sequences subsequent to a single integration event. The size of the genomic *BglII* fragment containing the cloned sequences is at least 13.6kb after the single integration event.

Restriction sites : P, *PstI*; C, *ClaI*; B, *BamHI*; X, *XbaI*; K, *KpnI*; Bg, *BglII*.



3kb ClaI fragment from p801

Genomic sequences homologous to the cloned sequences

YIp5 URA3 marker

pBR322 sequences from p801

Chromosomal DNA

YIp5 vector sequences

band detected in DNA from TDE/16A (6.7kb) it was predicted that the band in IT3 containing an insertion of YIpDE8 would be approximately 16kb in size. The observation of a band of between 15 and 20kb in the blot of genomic DNA from IT3 suggested that a single copy of YIpDE8 had integrated into the TDE/16A genome in this strain as the integration of two copies of the plasmid would have generated a band of a size greater than 25kb.

The genomic blot was stripped of the YIpDE8 probe DNA and reprobed with labelled pBR328 DNA (data not shown). pBR328 contains bacterial vector sequences that are homologous to YIpDE8 but no sequences that are homologous to yeast DNA. As expected the pBR328 probe detected only the 15-20kb fragment in the genomic DNA of IT3. This result supported the conclusion that the 15-20kb fragment observed in the DNA from IT3 was due to the integration of YIpDE8 at a site within the 6.7kb *BglII* fragment of TDE/16A.

The results of the Southern analysis further suggested that the gene that was homologous to the sequence cloned in p801 existed as a single copy in the TDE/16A genome. The lack of information concerning the exact size of the genomic *BglII* fragments containing probe homology prevented any conclusions as to whether or not the cloned sequence had escaped rearrangement during the process of molecular isolation.

Linkage Between the *dna26-1* Mutation and the Integrated Clone

The transformant strain IT3 (*MAT*a) was mated with strain DBY746 (*MAT*alpha *DNA26*$^{+}$ *ura3*). The resultant diploid was induced to sporulate and 25 tetrads were dissected that produced 4 viable spores. The germinated spore clones from each tetrad were screened

for uracil requirement and ability to proliferate at 36°C as well as adenine requirement and mating type (Table 4.11).

All 25 tetrads displayed a 2:2 pattern of segregation for the a and alpha mating types. A 2:2 pattern of segregation was also seen for the Ura^+ and Ura^- phenotypes in all 25 tetrads. This was expected as DBY746 contained a single *ura3-52* mutation and IT3 contained a single functional copy of the *URA3* gene derived from the integrated YIpDE8 plasmid. 24 out of 25 of the tetrads analysed displayed a 2:2 ratio of adenine requiring to adenine independent spores. One tetrad consisted of 3 adenine requiring and 1 adenine independent spore. IT3 contained the *ade2* mutation whilst DBY746 was adenine independent for growth. All tetrads were therefore expected to show a 2:2 segregation ratio for the adenine requiring and non-requiring phenotypes. The 3:1 segregation pattern observed in one tetrad was most likely due to a gene conversion event at the *ADE2* locus.

Table 4.11 also shows that 4 out of the 25 tetrads displayed a segregation ratio of $3ts^+:1ts^-$ spores whilst the spores from all the other tetrads displayed a ts^+ phenotype. The presence in 4 tetrads of segregants that displayed a temperature sensitive phenotype suggested that recombination had occurred between the site of integration of the cloned sequence and the *dna26-1* locus (discussed fully in section 4.4). Although this provided an inconclusive identification of the cloned gene, the data indicated that the *DNA26* gene and the cloned sequence were genetically linked. This evidence was therefore used in the physical mapping of the *DNA26* locus to one of the *S. cerevisiae* chromosomes.

MARKERS	MARKER SEGREGATION RATIOS	
	2:2	3:1
ts$^+$:ts$^-$	21	4
URA3:*ura3*	25	0
ADE2:*ade2*	24	1
*MAT*a:*MAT*alpha	25	0

TABLE 4.11 Segregation Data from the Cross Between IT3 and DBY746

The data are for the number of tetrads showing a particular segregation ratio for a range of markers. Strain IT3 was constructed by the integration of linearized plasmid YIpDE8 into the genome of the *dna26-1* mutant TDE/16A. The ability of segregants to proliferate at the restrictive temperature was determined by streaking cells for single colonies on YEPD agar and incubating at 36°C for 4 days.

PHYSICAL MAPPING OF THE CLONED GENE

Whole chromosomes from two strains of *S. cerevisiae* were physically separated by the CHEF system of pulsed field gel electrophoresis using the Bio-Rad CHEF-TM^{TR}II apparatus. When chromosome separation was carried out according to the manufacturer's instructions, 15 chromosomal bands were observed in the DNA preparations from strain YNN295 and 16 in the preparations from strain YPH148 (Plate 4.7). YNN295 contains 16 complete chromosomes and the doublet band observed in the preparation from this strain was assumed to be due to the comigration of chromosome XV and VII that is characteristic of this strain under the conditions employed (Chu *et al.*, 1986; CHEF-DR^{TM}II Instruction Manual - BioRad Labs.). Chromosome VII of strain YPH148 is fragmented into a large *RAD2*-proximal segment and a small *RAD2*-distal segment (Vollrath *et al.*, 1988). Under the conditions used the larger segment was assumed to be the band migrating ahead of chromosome XV and the smaller segment assumed to be the fastest migrating chromosomal band. The doublet band observed in chromosome preparations from YPH148 was assumed to be due to the comigration of chromosome XII and IV expected under the conditions employed (see Paddon & Hinnebusch, 1989).

In order to carry out a Southern hybridization analysis of chromosomal DNA the chromosomes from YNN295 and YPH148 were separated in adjacent lanes on a gel (Plate 4.8A). The chromosomes of strain YNN295 separated as anticipated but a high background prevented visualization of the chromosomes from YPH148. The cause of the background was unknown and was not as severe for any of the other simultaneously-prepared gel plugs that were subsequently examined. The chromosomal DNA was transferred to a nylon membrane and probed

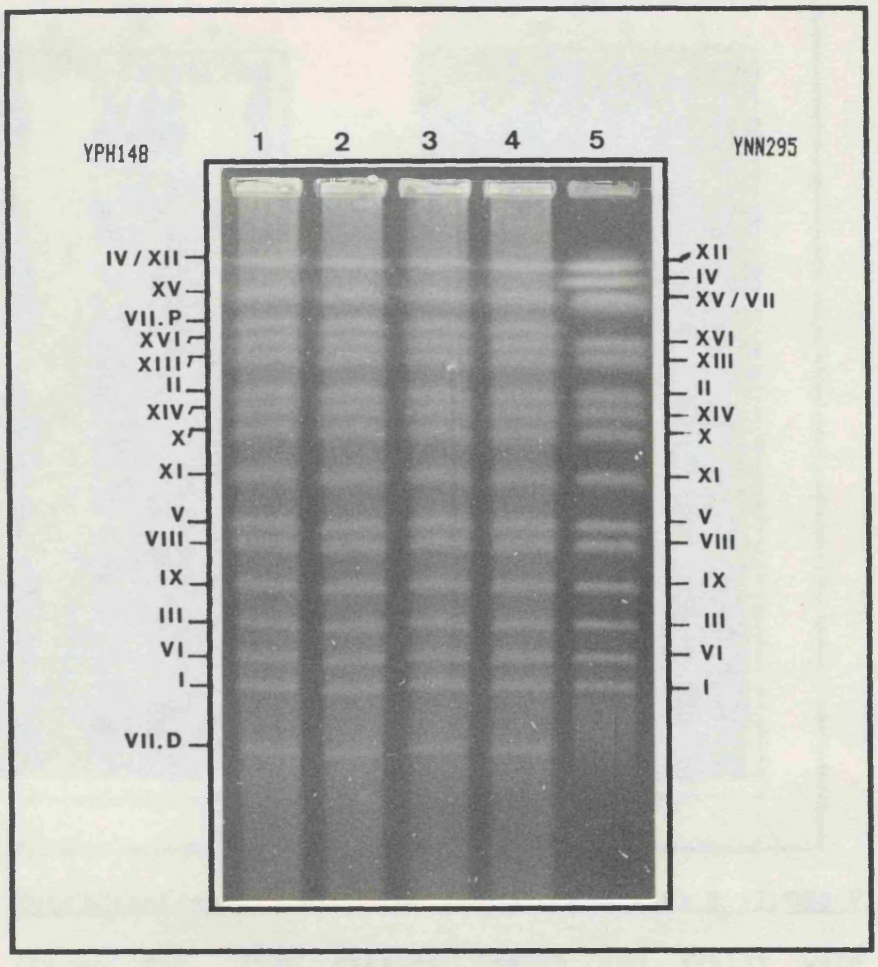


PLATE 4.7 Separation of S. cerevisiae Chromosomes

Chromosomes from strains YPH148 and YNN295 were separated by CHEF (according to manufacturers recommendations - see Materials & Methods, section 4.2). The migration distances expected under the conditions employed are indicated for the chromosome complement of both YPH148 (left-hand border) and YNN295 (right-hand border). Chromosome VII of strain YPH148 has been split into a *RAD2*-proximal (VII.P) and a *RAD2*-distal (VII.D) fragment (Vollrath *et al.*, 1988).

Lanes 1-4 : Chromosomes of strain YPH148.

Lane 5 : Chromosomes of strain YNN295.

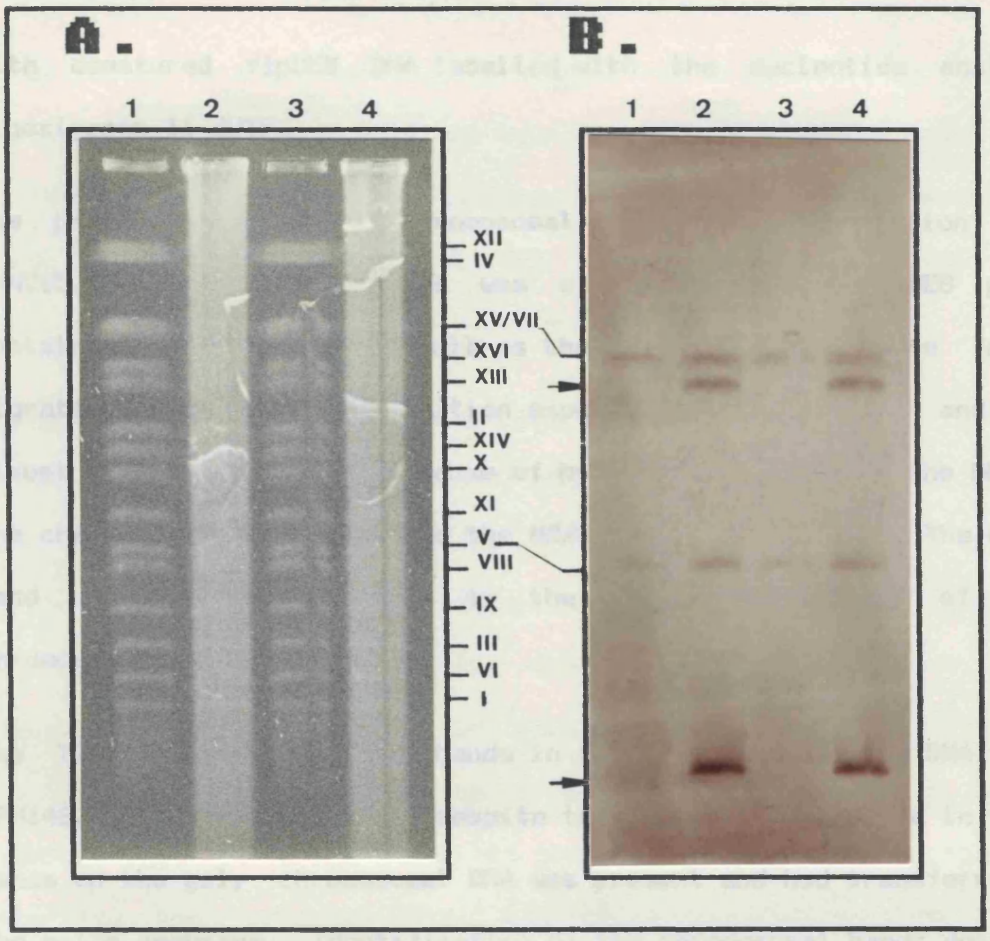


PLATE 4.8 Hybridization of Yeast Chromosomal DNA with a YIpDE8 Probe

Chromosomes from strains YNN295 and YPH148 were A, separated by CHEF under standard conditions (see section 4.2), and B, transferred to a nylon membrane and probed with digoxigenin-labelled YIpDE8 DNA. Lanes 1 and 3, chromosomal DNA from strain YNN295; Lanes 2 and 4, chromosomal DNA from strain YPH148. Only the expected positions for the YNN295 chromosomes have been labelled as the chromosomal bands from YPH148 were obscured on the gel by a high background of staining. Corresponding chromosomal bands on gel and membrane were identified by measuring migration distances from the position of the loading well. The arrows indicate probable positions of the higher molecular weight *RAD2*-proximal and lower molecular weight *RAD2*-distal chromosome VII fragments of YPH148.

with denatured YIpDE8 DNA labelled with the nucleotide analogue digoxigenin-11-dUTP.

The probe hybridized to 2 chromosomal bands in the preparation from YNN295 (Plate 4.8B). This was expected as the YIpDE8 probe contained the *URA3* gene as well as the genomic insert. The faster migrating band was at the position expected for chromosome V and was almost certainly detected because of hybridization between the DNA of the chromosomal *ura3* locus and the *URA3* marker on YIpDE8. The other band detected corresponded to the migration distance of the chromosome VII/XV doublet.

The YIpDE8 probe detected 4 bands in the lanes containing DNA from YPH148. This indicated that despite the background observed in these lanes on the gel, chromosomal DNA was present and had transferred to the nylon membrane. Identification of the chromosomal bands detected by the probe in this case was achieved by comparing the positions of the bands on the membrane with the migration distances of the YNN295 chromosomal bands on the gel. The probe detected a band corresponding to the migration distance of chromosome V which was again almost certainly due to the hybridization of homologous *URA3* sequences. The probe also detected bands corresponding to the migration distance anticipated for the smaller chromosome VII fragment (migrating faster than all other YNN295 chromosomes) and the migration distance predicted for the larger chromosome VII fragment (running between chromosomes XV and XVI of YNN295). The chromosome VII fragments in YPH148 were constructed by the integration into the chromosome of vector DNA containing sequences from the bacterial plasmid pBR322 (Appendix 1(F)). The pBR322 sequences of YIpDE8 were not removed before preparation of the probe and were therefore able

to hybridize to and detect the chromosome VII fragments from YPH148. In addition, the YIpDE8 probe hybridized to the chromosomal band corresponding to the predicted migration distance of chromosome XV. These results suggested that the cloned insert in YIpDE8 was homologous to sequences on chromosome XV.

Three further hybridization experiments were carried out to provide evidence to support the mapping of the cloned sequences to chromosome XV. Firstly a chromosome blot was probed with YIp5 vector DNA. Chromosomes from YNN295 were separated under the normal conditions (Plate 4.9A). The chromosomal DNA was transferred to a nylon membrane and probed with YIp5 DNA labelled with digoxigenin-11-dUTP. The YIp5 probe detected only the chromosome V DNA as expected (Plate 4.9B). This confirmed that the probe sequences responsible for detecting the chromosome XV/VII doublet in preparations from YNN295 in the first experiment were yeast sequences confined to the insert in YIpDE8.

A further experiment was carried out to separate the chromosome XV and VII bands in a preparation from YNN295. The run time for chromosome separation was extended to 40hr and the CHEF parameters were altered to give a single step separation (see section 4.2). Under these conditions all the chromosomes that migrated faster than chromosome V (but were also previously undetected by the YIpDE8 probe) were lost from the gel (Plate 4.10A). Chromosomes from YPH148 were also separated on the same gel with the resolution of chromosomes XII and IV from this strain being much greater than under the previous conditions. The large chromosome VII band from YPH148 also migrated much further than the complete chromosome VII band from YNN295 under the new conditions. The chromosome XV bands from the

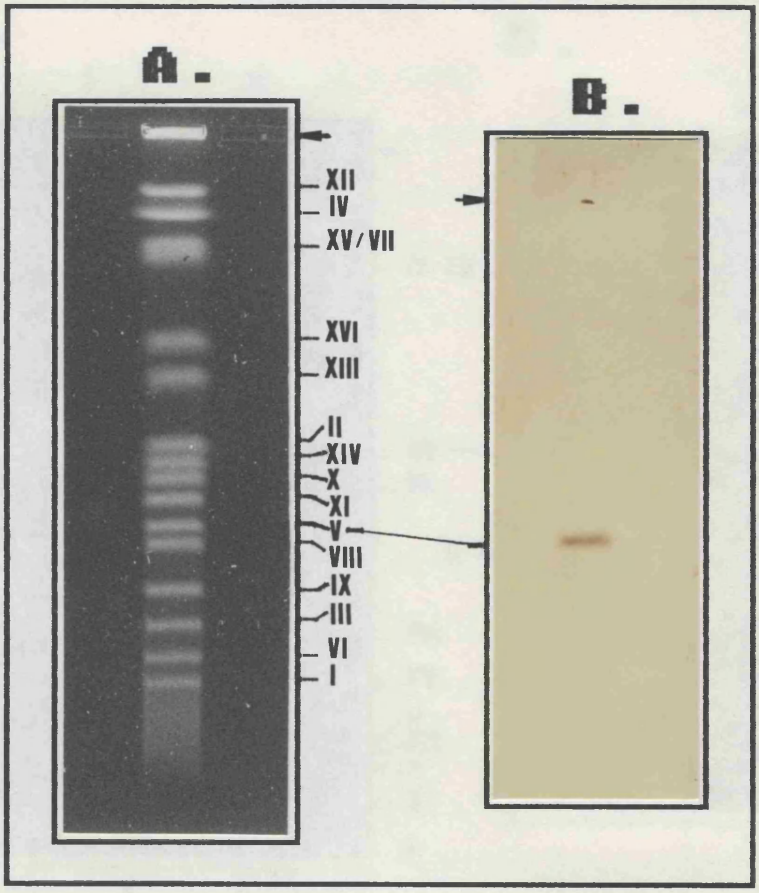


PLATE 4.9 Hybridization of YNN295 Chromosomal DNA with a YIp5 Probe

Chromosomes of strain YNN295 were A, separated by CHEF under standard conditions (see section 4.2), and B, transferred to a nylon membrane and probed with digoxigenin-labelled YIp5 DNA. The expected chromosomal migration distances for the conditions used are indicated on the gel. The arrows indicate the position of the loading well. Chromosome V was identified on the membrane on the criterion of its migration distance from the loading well.

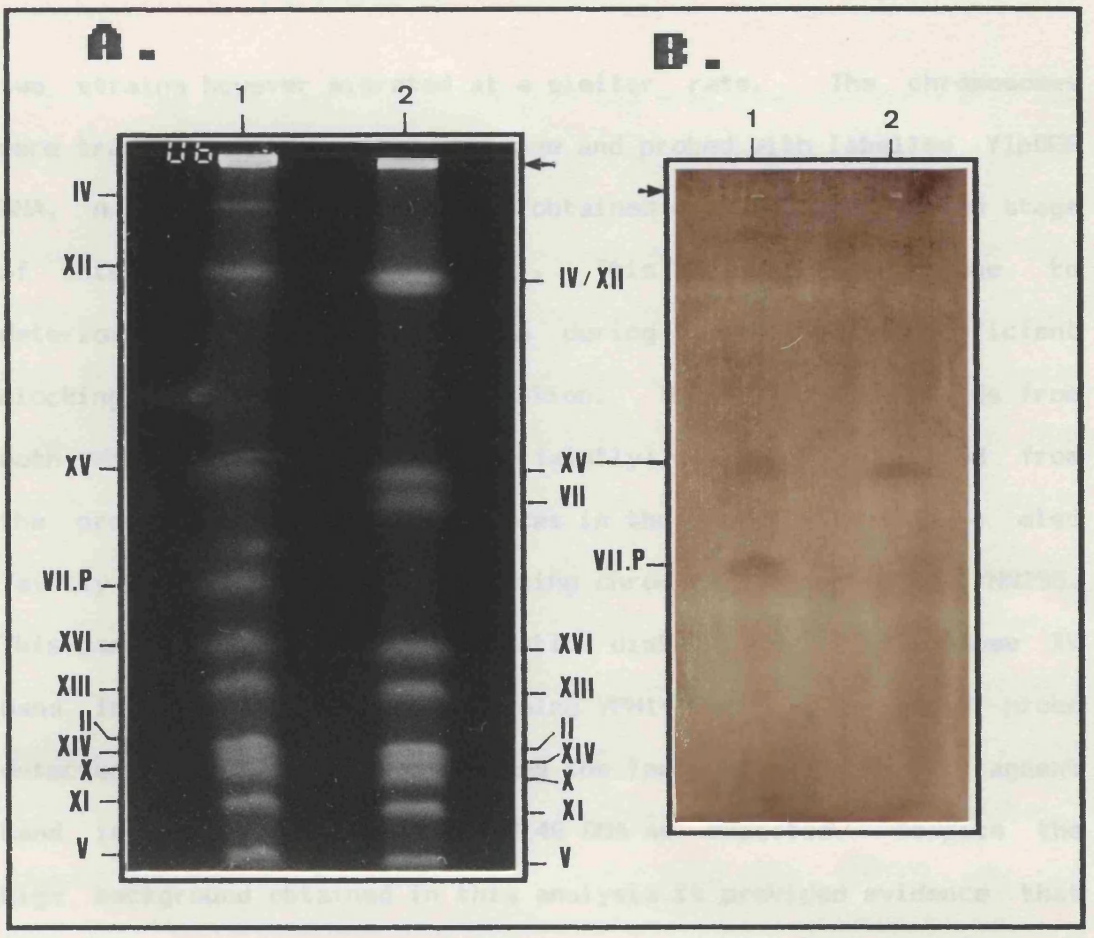


PLATE 4.10 Hybridization of Separated Chromosomes with YIpDE8 DNA

Chromosomes of strain YPH148 and YNN295 were A, separated by CHEF (40hr run-time, see section 4.2), and B, transferred to a nylon membrane and probed with digoxigenin-labelled YIpDE8 DNA. Lane : 1, YPH148; 2, YNN295 chromosomes. The probable migration distances under the conditions employed are indicated on the gel for the YPH148 (left-hand border) and YNN295 (right-hand border) chromosomes. VII.P - *RAD2*-proximal chromosome VII fragment from YPH148. Arrows indicate positions of loading wells used to measure migration distances.

two strains however migrated at a similar rate. The chromosomes were transferred to a nylon membrane and probed with labelled YIpDE8 DNA. A very high background was obtained during the detection stage of this analysis (Plate 4.10B). This may have been due to deterioration of the probe DNA during storage or insufficient blocking of non-specific hybridization. The chromosome V bands from both YNN295 and YPH148 were (very faintly) detected as expected from the presence of the *URA3* sequences in the probe. The probe also faintly detected a slower migrating chromosomal band from YNN295. This band displayed the same migration distance as the chromosome XV band in the adjacent lane containing YPH148 DNA. The YIpDE8 probe detected both the chromosome XV and the large chromosome VII fragment band in the lane containing YPH148 DNA as expected. Despite the high background obtained in this analysis it provided evidence that the cloned sequences in YIpDE8 were hybridizing to sequences on chromosome XV.

To confirm that the slower migrating chromosome from the doublet band in YNN295 preparations was chromosome XV a blot of YNN295 chromosomes was probed with the *WHI2* gene which has been previously mapped to chromosome XV (Mortimer *et al.*, 1989). Plasmid pSG6 (a gift from Peter Sudbery) consists of a 2.725kb fragment containing the *WHI2* gene cloned into the *Bam*HI site of the pBR322 derivative pAT153 (Saul & Sudbery, 1985; Twigg & Sherratt, 1980). pSG6 DNA was prepared by the maxi-prep. method and its structure checked by digestion with *Pst*I (data not shown). The plasmid DNA was then labelled with digoxigenin-11-dUTP and used to probe the chromosomal DNA from YNN295 and YPH148 that had been separated by CHEF electrophoresis during a 40hr run time (Plate

4.11A). The pSG6 probe detected both the predicted chromosome XV and large chromosome VII fragment band from YPH148 (Plate 4.11B).

Detection of the chromosome VII fragment was again expected from the presence of pBR322 sequences within the DNA of both the chromosome VII fragment and the probe. The probe also detected a chromosomal band from YNN295 that displayed the same migration rate as the chromosome band in the YPH148 preparation that had been provisionally designated as corresponding to chromosome XV in the previous analyses. These bands of identical migration rate were concluded to correspond to DNA from chromosome XV that hybridized to the *WHI2* sequences in the probe. The pSG6 probe also faintly detected the chromosome V band from YNN295. The reason for this was unclear, especially as the adjacent chromosome V band from YPH148 was not detected.

From the above hybridization experiments it was concluded that the insert in YIpDE8 contained a DNA sequence that was homologous to sequences present on chromosome XV of *S. cerevisiae*. As the sequences contained in the insert in YIpDE8 had been shown to be linked to the *dna26-1* locus it was further concluded that the *DNA26* locus was located on chromosome XV.

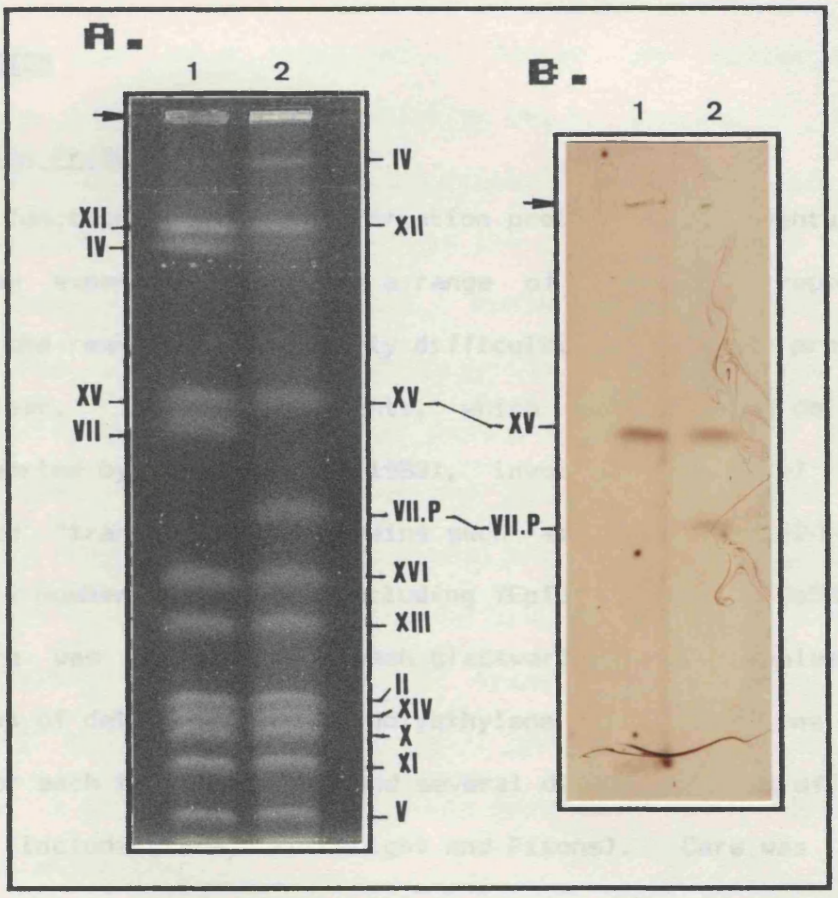


PLATE 4.11 Hybridization of *S. cerevisiae* Chromosomes with *WHI2* DNA

Chromosomal DNA was A, separated by CHEF (40hr run-time, see section, 4.2), and B, transferred to a nylon membrane and probed with digoxigenin-labelled pSG6(*WHI2*) DNA. Lanes : 1, YNN295 DNA; 2, YPH148 DNA. The expected migration distances have been indicated for the YPH148 chromosomes (right-hand border of gel) and for the YNN295 chromosomes when differing from YPH148 (left-hand border of gel). VII.P. - *RAD2*-proximal chromosome VII fragment from YPH148. The arrows mark the positions of the loading wells from which the migration distances of chromosomes were measured prior to photography.

4.4 DISCUSSION

Transformation Protocols

Although a functional yeast transformation protocol was eventually developed by experimentation with a range of previously reported conditions the reasons for the early difficulties with this process remain unclear. Initial experiments, which were all based on the protocol reported by Ito *et al.*, (1983), involved a number of well characterized "transformable" strains such as DBY746, LL-20 and MD40/4C and a number of plasmids including YEp13, pJDB248, YCp50 and pMA3A. Care was taken to acid wash glassware and disposables to remove traces of detergent. Fresh polyethylene glycol solutions were prepared for each transformation and several different makes of PEG were tried (including BDH, Koch-Light and Fisons). Care was also initially taken to purify plasmid DNA in ceasium chloride gradients and to only use yeast cells that were in the exponential phase of growth. Transformation of cells in the presence of Ca^{++} ions instead of Li^{+} ions was also attempted (Bruschi *et al.*, 1987). No transformants were obtained until the protocol essentially outlined in the section 4.2, Materials and Methods, employed. The final protocol contained only minor modifications to the protocol reported by Ito *et al.*, (1983). The major difference was that the final concentration of competent cells to which plasmid DNA was added was an order of magnitude higher than that specified in the original protocol. However, similar concentrations of cells had been used in the transformation attempts that failed. It was concluded that some critical factor had been serendipidously incorporated into the final transformation protocol that had been omitted in earlier procedures.

The final protocol for transformation in the presence of lithium ions

may not have been fully optimized. During the optimization experiments a competent cell concentration was not reached at which the amount of added plasmid DNA became limiting for increase in the number of transformants generated. Also the conditions of the heat pulse were not extensively optimized. Furthermore the effect of single stranded carrier DNA on transformation efficiency was not properly investigated. The effect of double stranded herring sperm DNA was however observed to have no effect when added at concentrations ranging from 0.1 to 100μg per 100μl of competent cells (data not shown). Nevertheless the protocol that was finally derived permitted a high enough transformation efficiency (approximately 10^3 transformants per μg of plasmid DNA) to make the isolation of a gene from a plasmid-borne gene library achievable, which was the major goal.

Isolation of Plasmids from the Gene Libraries in pMA3A and YCp50

Twelve transformants generated from the pMA3A-based gene library were intially isolated that were capable of proliferation at 36°C. Seven of these transformants however displayed loss of the ts^+ phenotype at various frequencies when grown on selective or non-selective medium. It is possible that the latter transformants contained plasmids carrying a weak suppressor of the *dna26-1* mutation. The ability of these plasmids to suppress the temperature sensitive phenotype of TDE/16A may therefore have been sensitive to their dosage in the cell. The loss of the ts^+ phenotype in these transformants may have thus been due to fluctuations in plasmid copy number. Too low a copy number may have provided insufficient gene dosage for suppression of the *dna26-1* mutation. It is also possible for plasmids based on the pMA3A vector that excessive gene dosage may have been the cause of

instability in some cases due to toxic effects on the cell. Further examination of these transformants at semi-permissive temperatures may prove to be profitable for the isolation of dosage suppressors of *dna26-1*.

All five transformants obtained from the pMA3A-based library that showed coincident mitotic instability of both the ts$^+$ and Leu$^+$ phenotypes were found to contain apparently identical plasmids. The pMA3A vector has a very high copy number in yeast cells potentiating the isolation of dosage suppressors from a gene library based on this plasmid. However the isolation of the same or similar genomic sequence from five independent transformants when selecting for functional complementation of the *dna26-1* mutation was strongly suggestive of the isolation of the wild type *DNA26* gene.

Two transformants capable of proliferating at 36°C were also initially isolated from the gene library constructed in the YCp50 vector. These transformants however displayed an extremely high frequency of loss of the ts$^+$ phenotype. It is therefore unlikely that the plasmids in these transformants contained the *DNA26* gene. It is uncertain why no plasmids capable of stably suppressing the *dna26-1* mutation were isolated from this gene library even though more Ura$^+$ transformants were screened than Leu$^+$ transformants from the pMA3A library. The gene libraries were constructed in a similar fashion with 10-15kb partial *Sau*3A fragments being inserted into the relevant vectors. It is possible that during the storage and amplification of the YCp50-based library in *E. coli* the plasmids carrying *DNA26* sequences may have become under-represented.

Site of Integration of the Cloned Sequence

In order to discover the degree of linkage between the cloned sequence and the *DNA26* locus the integration of the clone was targetted to its homologous site in the *dna26-1* mutant genome of TDE/16A. When the resultant transformant IT3 was crossed with the *DNA26*$^+$ strain DBY746 and the resultant diploid induced to sporulate, 4 out of the 25 tetrads examined exhibited a segregation ratio of 3ts$^+$:1ts$^-$ spores. This was not the segregation pattern expected for the situation in which the clone had integrated at the *dna26-1* locus of TDE/16A; evidence for which would have been obtained by the absence of ts$^-$ segregants. Neither was the segregation pattern indicative of the integration of the clone at a genomic site that was unlinked to the *dna26-1* locus. This alternative event would have been indicated by a high proportion of tetrads displaying a 3ts$^+$:1ts$^-$ and 2ts$^+$:2ts$^-$ ratio of segregants and was clearly not the pattern observed. Rather, the pattern of segregation observed was that expected if the cloned sequence had integrated at a genomic site that was closely linked to the *dna26-1* locus. By this argument, the 4 asci containing a single ts$^-$ spore were generated by low frequency, single recombination events occurring in the interval between the site of integration of the clone and the *dna26* locus. On this assumption the theoretical distance between the two loci, calculated from the segregation data, was 8.0cM.

A conclusion on the basis of the above data that the cloned sequence is a closely linked but extragenic suppressor of the *dna26-1* mutation appears to conflict with some of the other results obtained. Five plasmids independently isolated from the gene library in pMA3A apparently contained the same genomic insert capable of functionally

complementing the *dna26-1* mutation. The multiple isolation of this genomic sequence suggested that it was the wild type copy of the *DNA26* gene. Furthermore the sub-cloning of the cloned sequence to the low copy vector YCp50 revealed that the clone was capable of suppressing the *dna26-1* mutant phenotype at a dosage of 1 or 2 copies per cell. This ability was verified by the integration of a single extra copy of the cloned sequence into the TDE/16A genome. The integration of YIpDE8 produced stable transformants that could proliferate with apparently wild type vigour (as judged by colony formation on plates at 36°C). This low copy function is uncharacteristic of a dosage suppressor.

Other explanations can be offered for the observation of ts^- progeny arising from the cross between IT3 and DBY746. It is possible that the 3.75kb *ClaI* genomic fragment sub-cloned to YCp50 and YIp5 contained two functional genes complementing two closely linked temperature sensitive mutations in TDE/16A. During the construction of TDE/16A some tetrads displaying a $3ts^-:1ts^+$ ratio of spores were observed suggesting the presence of a temperature sensitive mutation other than the *dna26-1* mutation. Two such closely linked mutations may not have been separated by the three rounds of backcrossing carried out during the construction of TDE/16A. The data obtained from the Tn5 mutagenesis provide evidence against the existence of multiple suppressors within the sub-cloned fragment however, as single Tn5 insertions were shown to cause disruption of the suppression of the temperature sensitive phenotype of TDE/16A. The uncertainty of whether TDE/16A contains two closely linked temperature sensitive mutations may however require clarification by further backcrossing of TDE/16A to a wild type strain. A wild type

strain other than MD40/4C may be required for such experiments to eliminate the possibilty that a temperature sensitive mutation may have been introduced into the *dna26-1* mutant from MD40/4C itself.

DBY746 was used as the wild type strain in the linkage experiment as the only available *MAT*alpha ts$^+$ strain that carried the appropriate auxotrophic markers. DBY746 was originally constructed for its high transformability rather than for characteristics of wild type proliferation. It is therefore possible that the temperature sensitive segregants arising from the cross between IT3 and DBY746 derived from the uncovering of temperature sensitive mutations in DBY746. By this argument, DBY746 may contain temperature sensitive mutations that are suppressed in the DBY746 genetic background but are revealed during recombination with the IT3 genome. A test of this hypothesis might be provided by screening for temperature sensitive progeny during the serial backcrossing of DBY746 to another wild type strain of better studied growth characteristics.

It has been proposed that suppressed temperature sensitive mutations may be present in apparently wild type populations which can be revealed during recombination of yeast genomes (Hanic-Joyce, 1985). This phenomenon was revealed during the mapping of the *cdc60* to *cdc65* mutations by the *rad52*-mediated chromosome loss technique. In the latter study the temperature sensitive *cdc* mutations were discerned from spontaneously arising temperature sensitive mutations by classical complementation analysis after construction of the *cdc* strains in the two alternative mating types.

Finally, it is uncertain what effect the integration of the YIpDE8 vector containing the cloned sequence may have itself had on the

recombination data. If the *dna26-1* mutation is a point mutation the integration of YIpDE8 at the *dna26-1* locus would be expected to generate one mutant copy of the *DNA26* gene and one wild type copy intervened by YIp5 vector sequences. An additional 9.29kb (the size of YIpDE8) is therefore added to the genome of TDE/16A by this event which may allow scope for recombination between the two allelic loci or may even promote deletion of *DNA26* sequences.

Location of the *DNA26* Locus on Chromosome XV

Integration of the cloned sequence was targetted to its homologous genomic site by linearization of the YIpDE8 construct within the insert DNA prior to transformation. The site of integration was shown by tetrad analysis to be linked to the *dna26-1* locus and using this information the *DNA26* locus was physically mapped to chromosome XV. The segregation of the *ade2* mutation was also fortuitously monitored during the tetrad analysis. The *ade2* mutation has been previously mapped to chromosome XV (Mortimer *et al.*, 1989). However the *URA3* marker on the integrated YIpDE8 plasmid displayed no linkage to the *ade2* mutation from strain IT3 during this analysis. The number and classes of tetrads observed in the case of these two markers were 2PD:7NPD:15TT indicating a theoretical genetic distance between the two loci of 118.75cM. During the construction of TDE/16A a similar result was observed for the pattern of segregation of the ts^- phenotype and a single *ade2* marker (data not shown). In this cross the ratio of ascal classes was 3PD:3NPD:12TT corresponding to a theoretical genetic distance of 83.3cM. This result is not necessarily anomalous as chromosome XV is the third largest chromosome of *S. cerevisiae*. It probably indicates that the *DNA26* and *ADE2* loci are distantly separated on chromosome XV. A similar

result was obtained during the mapping of the *CDC72* and *CDC73* loci which displayed no apparent linkage during tetrad analysis despite being physically mapped each to chromosome XV (Reed *et al.*, 1988).

Several genes are located on chromosome XV for which mutations have been identified that produce a *cdc* phenotype. The two mutations with loci on this chromosome that most strikingly resemble the *dna26-1* mutant phenotype are the *prt1* mutation and the *gcd1* mutation. The *prt1-63* allele was originally isolated as a START mutation (Bedard *et al.*, 1981) and all *prt1* mutations are able to cause an arrest of cell cycle progression in G1 under the appropriate restrictive conditions (Hanic-Joyce *et al.*, 1987a). Similar to the *dna26-1* mutation, mutations in the *PRT1* gene cause a rapid decrease in the rate of protein synthesis under restrictive conditions. The *dna26-1* mutation has been shown to complement the *prt1-1* and *prt1-63* mutant alleles in a diploid strain (Davies, 1985; Green, 1986 - but see section 5.1).

The *gcd1-1* mutation also causes a G1 arrest phenotype and a rapid decrease in the rate of protein synthesis when mutant cells are shifted from 23°C to 37°C (Hill & Struhl, 1988). The *GCD1* gene product contributes to the derepression of the *GCN4* gene under conditions of amino acid starvation. It has been suggested that the *GCD1* gene product may be a translation termination factor. No complementation tests have been carried out involving a *gcd1-1* and a *dna26-1* mutant. The restriction map of the cloned *GCD1* gene is dissimilar to that deduced for the insert in plasmid p801 in this study.

Two other genes located on chromosome XV are the *CDC64* and *CDC66* genes. The *cdc64-1* mutation was originally isolated as a conditional

mutation that rescued the lethal effect of a *cdc4* mutation by arresting cells in G1 prior to the stage of *cdc4* arrest (Bedard *et al.*, 1981). The *cdc64-1* mutation has been characterized as a Class II START mutant that causes a mating defect and a rapid decrease in the rate of protein synthesis under restrictive conditions. The *cdc66-1* mutation was identified as causing an increase in cell volume under restrictive conditions due to a defect in the formation of new buds (Prendergast *et al.*, 1990a). The *CDC66* gene encodes an isoform of myosin heavy chain and is involved in the directed movement of secretory vesicles containing new cell wall material to the site of the growing bud (Prendergast *et al.*, 1990b). The nuclear cycle proceeds unimpaired in *cdc66* mutants incubated at the restrictive temperature. The *dna26-1* mutation has been shown to complement both the *cdc64-1* and *cdc66-1* alleles in a diploid cell incubated at 36°C (Davies, 1985).

The *RAS1* gene is also located on chromosome XV. No complementation tests have been carried out between a *dna26-1* and a *ras1* mutant. However the *DNA26* and *RAS1* genes are unlikely to be allelic as *RAS1* function is thought to be redundant in the presence of a functional *RAS2* gene (Kataoka *et al.*, 1984). The *RAS1* gene is also tightly linked to the *ADE2* locus, unlike the *dna26-1* mutation. Furthermore, it has been shown that the *dna26-1* mutation is not suppressed by the *RAS2* gene carried on a multi-copy plasmid (see section 2.3). The *IRA2* gene, which is thought to promote the GTPase activity of Ras proteins, is also located on chromosome XV (Tanaka *et al.*, 1990a). Disruption of the *IRA2* gene causes sensitivity to heat shock and nitrogen starvation and defective sporulation in diploids.

The *CDC21* and *CDC31* genes have been mapped to chromosome XV. The

cdc21 mutation encodes a mutant form of thymidylate synthetase and causes a defect in DNA synthesis (Pringle & Hartwell, 1981). The mutant cells arrest with a single bud at the restrictive temperature and produce petites at high frequency. The *cdc31* mutation causes a defect in spindle pole body duplication resulting in the formation of a unipolar spindle (Byers, 1981). Cells arrest with a single large bud at the restrictive temperature and undergo polyploidization at high frequency due to the continuation of DNA synthesis. The *dna26-1* mutation has been shown to separately complement both the *cdc21* and *cdc31* mutations in a diploid cell at 36°C (Davies, 1985).

Other potential alleles of *DNA26* on chromosome XV include *WHI2* and *PDE2*. Mutations in the *WHI2* gene cause an inability of mutant cells to enter stationary phase upon carbon starvation (Sudbery *et al.*, 1980; Saul *et al.*, 1985). The *PDE2* gene encodes the high affinity phosphodiesterase of *S. cerevisiae* and when expressed on a high copy vector can suppress the effects of a $RAS2^{val19}$ mutation (Sass *et al.*, 1986). The *dna26-1* mutation, if allelic to the above genes, would therefore be expected to encode a hyperactivated mutant gene product in each case.

CHAPTER 5 : CONCLUSIONS

Comparison of the Cloned Sequence in p801 with Other Cloned Genes

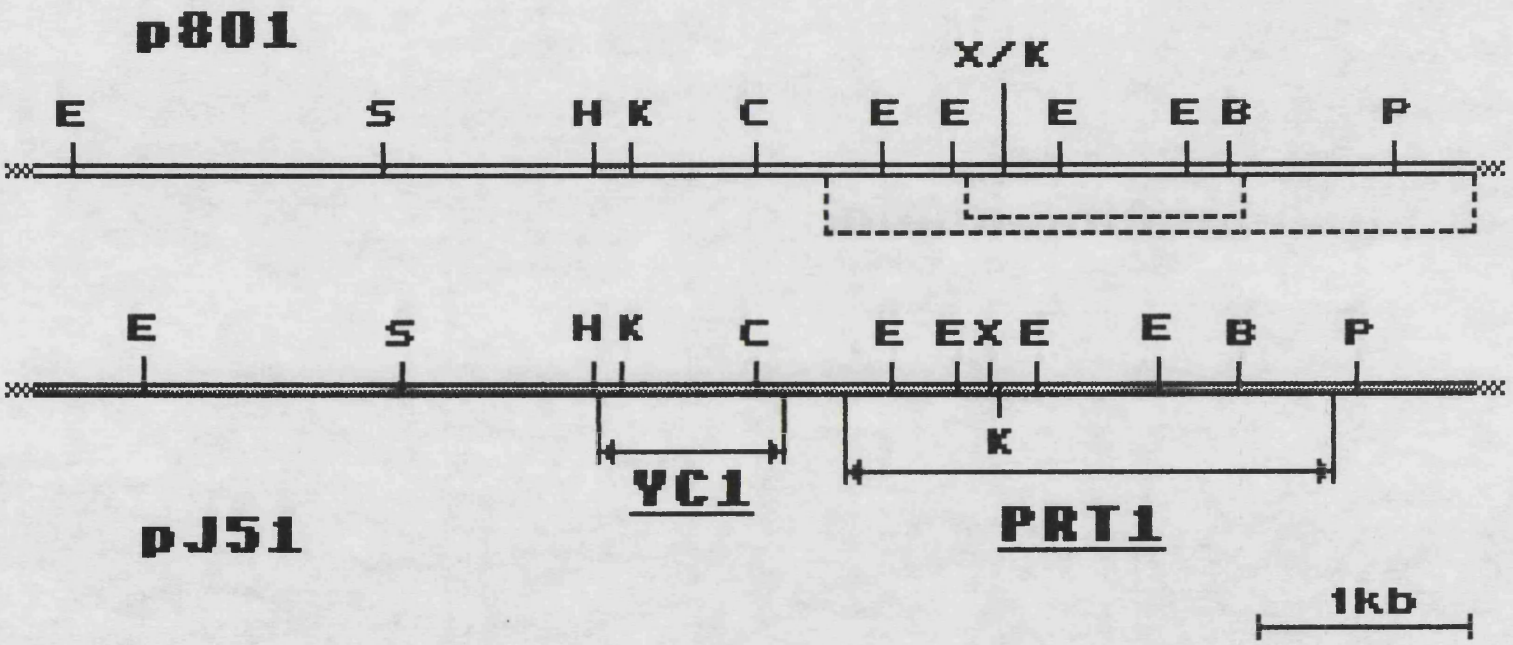
The mapping of the *dna26-1* locus to chromosome XV within the *S. cerevisiae* genome prompted a comparison of the cloned fragment in p801 with previously cloned genes that have also been mapped to chromosome XV. This was carried out by comparing the restriction map of various genes thought to be involved in cell cycle regulation with the map of the genomic fragment cloned in plasmid p801.

The *PRT1* gene is located on the right arm of chromosome XV (Hanic-Joyce, 1985). Comparison of the insert in p801 with the cloned sequences surrounding and including the *PRT1* gene (Hanic-Joyce *et al.*, 1987b) revealed that the two sequences contained a striking similarity in the number and location of restriction sites for *Eco*RI, *Cla*I, *Hind*III, *Bam*HI, *Pst*I, *Kpn*I, *Xba*I and *Sal*I (Figure 5.1). Some variation in the spacing of the restriction sites in the cloned inserts of plasmid pJ51 (containing the region surrounding *PRT1*) and plasmid p801 was observed. This variation however is most likely due to an inaccuracy in the gel electrophoretic analysis of the restriction fragments from p801. The cloned sequence in p801 therefore appears to encompass the *PRT1* gene.

A close comparison of the cloned fragment from p801 with the sequences surrounding the *PRT1* gene, suggested that the cloned fragment contains sequences neighbouring the *PDE2* gene. The *PDE2* gene is centromere-proximal to the *PRT1* locus on chromosome XV (Sass *et al.*, 1986), with the end of the *PRT1* open reading frame (ORF) and the start of the *PDE2* ORF being separated by approximately 1kb of genomic DNA. The insert in p801 was estimated to extend for only approximately 0.5kb beyond the putative termination point of the *PRT1* ORF. It is therefore unlikely that the insert in p801 confers *PDE2*

FIGURE 5.1 Comparison of the Restriction map of the Genomic Inserts in p801 and pJ51

The figure shows a comparison of the genomic inserts in p801 (upper) and pJ51 (lower). pJ51 is known to contain the *PRT1* and *YC1* genes (locations arrowed) and adjacent genomic sequences (Hanic-Joyce *et al.*, 1987b; Fujiwara *et al.*, 1990). Where possible, the restriction map of pJ51 was reproduced from the nucleotide sequence data for the *PRT1* and *YC1* genes, but otherwise from the original map. For the purposes of comparison the two restriction maps are centered on the *ClaI* site. The dotted lines below the p801 map represent the upper and lower limits for the size of the *dna26-1*-suppressing sequence as deduced from the Tn5 mutagenesis data. E, *Eco*RI; S, *SalI*; H, *HindIII*; K, *KpnI*; C, *ClaI*; X, *XbaI*; B, *BamHI*; P, *PstI*. The adjacent *KpnI* and *XbaI* sites in p801 were not resolved during the restriction mapping of this plasmid and are therefore labelled *(X/K)*.


p801
X/K
E
S
H K
C
E E
E
E B
P
E
S
H K
C
E E X E
E
B
P
K
YC1
PRT1
pJ51
1kb

gene function. The insert in p801 apparently also contains the *YC1* gene which lies centromere distal to *PRT1* and encodes a proteasome component (Fujiwara *et al.*, 1990). The 3kb *ClaI* fragment that was subcloned from p801 contained exactly 375bp upstream of the most centromere distal methionine codon that begins the largest *PRT1* ORF. This region separates the *PRT1* and *YC1* ORF's (which read in the same direction on the chromosome). Tn5 insertions into the 375bp stretch did not disrupt the activity causing suppression of the *dna26-1* mutation.

The Effects of Mutations in the *PRT1* Gene

The first identification of a mutation in the *PRT1* gene was made during a screen for temperature sensitive mutants defective in protein synthesis (Hartwell, 1967; Hartwell & McLaughlin, 1968). The mutant strains ts-187 and ts-171 were shown to contain single allelic mutations causing a rapid and severe decrease in the rate of incorporation of radiolabelled amino acids into protein. This apparent defect in protein synthesis was shown to occur in the absence of any adverse effect on energy metabolism or membrane function.

The *prt1-1* mutation in strain ts-187 was shown to cause a dissociation of polyribosomes into monoribosomes within two minutes of a shift of the mutant culture to the restrictive temperature (Hartwell & McLaughlin, 1969). The dissociation of polyribosomes was shown to be accompanied by the release of nascent proteins into the cytoplasm of the mutant cells. These observations indicated that the *prt1-1* mutation causes a defect in translational initiation rather than polypeptide chain elongation or termination. Further analysis

of the *prt1-1* mutation was carried out by investigation of its effect on translation *in vitro* using cell free extracts (Feinberg *et al.*, 1982). The *prt1-1* mutation was shown to cause a temperature sensitive defect in the *in vitro* translation of exogenous natural mRNA, but not in the translation of a synthetic polyuridylic acid template; translation of poly(U) reflects the efficiency of the elongation component of translation. Individual reactions occurring in the initiation of translation were examined in cell free extracts from a wild type strain and a *prt1-1* mutant. These experiments revealed a defect in the interaction between the [eukaryotic initiation factor-2(eIF-2).Met-tRNA$_f$.GTP] ternary complex and the 40S ribosomal subunit in the mutant extracts at 36°C.

It has been suggested by Moldave & McLaughlin, (1988) that the genetically altered component in ts-187 is initiation factor-3 (eIF3). eIF3 has been isolated from rat liver cells (Thompson *et al.*, 1977) and yeast (Moldave & McLaughlin, 1988) and shown to consist of a complex of 7-10 polypeptide chains of different molecular weights. The eIF3 complex binds to 40S ribosomal subunits to form an obligatory precursor of the 40S preinitiation complex formed by the subsequent binding of the [eIF2.Met-tRNA$_f$.GTP] ternary complex and mRNA. eIF3 is thought to possess a ribosomal subunit anti-association activity preventing the interaction of the 40S and 60S ribosomal subunits before the completion of the 40S preinitiation complex (Moldave, 1985). The eIF3 activity purified from ts-187 extracts has been shown to be thermolabile (Moldave & McLaughlin, 1988).

A further allele of *PRT1* was identified in a screen for mutations that spared cells from the lethal effect of nystatin, a polyene

antibiotic that disrupts the cell membrane of growing cells (Thonart *et al.*, 1976). The mutant strain ts-1564 containing the *prt1-1564* allele was found to be conditionally defective for RNA synthesis. It is possible that the defect in this mutant resulted from the stringent response arising from a protein synthesis defect (Warner & Gorenstein, 1978).

The nucleotide sequence of the *PRT1* gene has been determined (Hanic-Joyce *et al.*, 1987b). It consists of a 763 codon open reading frame (ORF) which could code for a protein of M_r 88137. The gene is essential for mitotic growth and bears no amino acid sequence similarity to any other known protein. The Prt1 protein contains an 11-nucleotide sequence within its ORF which is similar to the HOMOL1 and RPG consensus sequences found upstream of most ribosomal protein genes and also upstream of the gene encoding the yeast elongation factor-1alpha (Teem *et al.*, 1984). These sequences are thought to be important for transcriptional regulation of gene expression (Rotenberg & Woolford, 1986). The codon usage in the *PRT1* gene suggests that it is moderately expressed. The amino acid composition is typical of soluble proteins and contains six Asn-X-(Ser/Thr) tripeptides which could serve as sites for asparagine-linked glycosylation. The *PRT1* gene also contains a 23-nucleotide sequence that resembles a motif that has been identified in several nucleotide binding proteins. Furthermore the Prt1 protein contains two consensus sequences that are potential targets for phosphorylation by the cAMP-dependent protein kinase.

An involvement of the *PRT1* gene in the control of the cell cycle was first revealed during a study of the *cdc63-1* mutation. This allele (also referred to as *prt1-63*) was identified in a screen for

temperature sensitive mutations causing conditional arrest of mutant cells in the G1 phase of the cell cycle (Bedard *et al.*, 1981). Classification of *cdc63-1* as a Class I START mutation was made on several criteria. Cell cycle arrest caused by the *cdc63-1* mutation was found by order of function mapping to occur at the alpha-factor sensitive step of the cell cycle. Moreover, during cell cycle arrest under restrictive conditions, *cdc63-1* mutant cells were shown to retain a high degree of conjugational competencey and to maintain a high rate of macromolecular synthesis. Additional analysis of the *cdc63-1* mutation revealed that it did not cause shmoo formation under restrictive conditions, and that it was not suppressed when homozygous in an a/alpha diploid cell.

An unexpected allelism between the *cdc63-1* and *prt1* mutations was revealed during the mapping of the *cdc63-1* mutation to the *S. cerevisiae* genome (Hanic-Joyce, 1985). Whilst *cdc63-1* was originally identified as causing a concerted arrest of mutant cells at START, early characterization of the *prt1-1* mutant phenotype under semi-permissive conditions (29°C) suggested that the effect of the *prt1* mutation was to cause merely a delay in the performance of START (Hartwell & Unger, 1977). The delay in the execution of START at 29°C was accounted for by a delay in the attainment of the minimum cell size for cell cycle initiation caused by the protein synthesis defect. However the use of alternative restrictive temperatures for the arrest of a number of mutants, each containing a different mutant allele of *PRT1*, has revealed that all known *prt1* mutations can under appropriate conditions cause a concerted arrest of cells at START (Hanic-Joyce *et al.*, 1987a). At 36°C, mutant cells carrying either of the *prt1-1*, *prt1-2*, *prt1-3* or *prt1-1564* mutations cease

proliferation abruptly, arresting at all stages of the cell cycle. However at the lower temperature of 34°C these same mutants display a concerted arrest of cell cycle progression at START. At the higher temperature the severe inhibition of protein synthesis apparently prevents the cells from completing the current cell cycle whilst at the lower temperature the mutant cells presumably retain a sufficient biosynthetic capacity to complete the cell cycle before arresting at START. The latter capacity is similar to that displayed by the *prt1-63* mutant at 36°C.

It has also been demonstrated that the mating ability of *prt1* mutants is dependent upon the restrictive temperature employed to arrest proliferation (Hanic-Joyce *et al.*, 1987a). After incubation at 36°C a *prt1-63* mutant retains a high mating competency. This competency is contrastingly poor in a *prt1-1* mutant incubated at 36°C, being similar to the mating ability of a *prt1-63* mutant after incubation at the higher restrictive temperature of 40°C. However at 32°C the *prt1-1* mutant mates well whilst also arresting at START. Mutations in the *PRT1* gene are thus capable of causing a Class I or Class II START arrest depending on the restrictive condition.

Mutations in the *PRT1* gene have been further examined by a comparison of their effects on cell cycle progression with the effects caused by the protein synthesis inhibitors cycloheximide and verrucarin A (Hanic-Joyce *et al.*, 1987a; Johnston & Singer, 1990). This study revealed that both the protein synthesis inhibitors were able to cause a decrease in the rate of cell division with cells spending a longer period in G1 at higher inhibitor concentrations. This G1 accumulation was again attributable to the longer time taken for cells to attain the minimum cell size for execution of START during

inhibition of protein synthesis. The same response was observed in *prt1-1* mutant cells at the semi-permissive temperature of 29°C. However, at no concentrations did the protein synthesis inhibitors cause a complete cessation of cell division at START as seen in the *prt1-1* mutant at 34°C and the *prt1-63* mutant at 36°C. High protein synthesis inhibitor concentrations caused cells to arrest at random positions in the cell cycle. The regulated cessation of cellular proliferation at START under conditions of protein synthesis inhibition was thus demonstrated to be a special property of the *prt1* mutation. Moreover, the results suggested a role for the *PRT1* gene in a regulatory mechanism at START distinct from the minimum size requirement for cell cycle initiation.

The *dna26-1* Mutation is a Probable Allele of *PRT1*

The finding that the cloned sequences in p801 capable of functionally complementing the *dna26-1* mutation were apparently identical to those encompassing the *PRT1* gene was unexpected from the results of previous complementation experiments. The *dna26-1* mutation had been shown to complement both the *prt1-1* and *cdc63-1* (*prt1-63*) mutations in a diploid cell incubated on YEPD agar at 36°C (Davies, 1985; Green, 1986; Richard Singer, personal communication). Positive complementation between the *dna26-1* and *prt1* mutations indicates that either, i) the two mutations are unlinked, ii) intragenic complementation is occurring or iii) the JL448 genetic background used in the above complementation tests somehow allows for suppression of the homozygous *dna26-1* mutant phenotype. If the second of the above hypotheses is correct then the mutant gene products encoded by the *dna26-1* and *prt1* mutations may be able to physically stabilize each other when present in the same cell. Such

stabilization is suggestive of a gene product that possesses a quaternary structure consisting of multiple associated subunits. This possibility is consistent with the suggestion that the Prt1 protein is a component of eIF3 (Moldave *et al.*, 1988). Alternatively the Prt1 protein may consist of multiple domains that carry out specific functions. The possibly allelic *dna26-1* and *prt1* mutations may by this argument encode mutant proteins with functionally complementing domains. Complementation tests involving the *dna26-1* mutant and mutants carrying alternative alleles of the *PRT1* gene (such as *prt1-2*, *prt1-3* and *prt1-1564*) may provide support for these arguments.

The observed complementation between *dna26-1* and the *prt1-1* and *prt1-63* mutations coupled with the data obtained from the integration experiment in this study suggests that the *PRT1* gene is a closely linked but extragenic suppressor of the *dna26-1* mutation. Arguing against this conclusion however is the fact that the apparently identical genomic fragment was isolated from the gene library in pMA3A in five independent transformation events. The cloned sequence was also shown to functionally complement the *dna26-1* mutation at low gene dosage, which is rare for a wild type extragenic suppressor. Furthermore the *dna26-1* mutant TDE/16A was shown to display the characteristics of cell cycle arrest in G1 and rapid inhibition of protein synthesis that are typical of cells carrying a mutation in the *PRT1* gene (Hanic Joyce *et al.*, 1987a). A mutation in the *PRT1* gene has also been reported to cause low spore viability during tetrad analyses (Thonart *et al.*, 1976), a trait observed during the strain construction of TDE/16A.

The protein synthesis defect caused by the *prt1-63* allele at 36°C has

been shown to be less severe than that caused by a *prt1-1* mutation at the same temperature (Hanic-Joyce *et al.*, 1987a). The apparent protein synthesis defect caused by the *dna26-1* mutation in TDE/16A at 36°C was intermediate in its severity to that observed for the latter two mutations (see Table 5.1). As an allele of *PRT1* the *dna26-1* mutation would therefore be expected to display either a random cell cycle arrest or a tight START arrest at 36°C due to the severe biosynthetic defect at 36°C. In fact, the mutant strain TDE/16A displayed a slow accumulation in G1 at 37°C without a complete cessation of cellular proliferation. The closest comparison with this response is the leaky START arrest reported for the *prt1-3* and *prt1-1564* mutants incubated at 34°C. It may prove instructive to investigate the mode of cell cycle arrest by the *dna26-1* mutant at a range of restrictive temperatures. Evidence supporting allelism between the *DNA26* and *PRT1* genes will be provided if, at higher restrictive temperatures than 37°C, the *dna26-1* mutant displays a tighter START arrest. As an allele of *PRT1* the *dna26-1* mutation should also cause an abrupt and random arrest of the cell cycle at very high restrictive temperatures (for example 40°C). Any conclusions from such an experiment might however be complicated by the fact that at temperatures approaching 40°C conditions become inhibitory for the proliferation of wild type cells of *S. cerevisiae*.

Further Analysis of the *DNA26* Gene

Despite the strong suggestive evidence that the *dna26-1* mutation is an allele of *PRT1*, further experiments will be required to provide absolute certainty of their common identity. Most simply, the *dna26-1* mutant may be crossed with a *prt1* mutant and the resultant diploid subjected to tetrad analysis. Evidence for allelism between

MUTANT ALLELE	RESTRICTIVE TEMPERATURE	TYPE OF ARREST	MATING EFFICIENCY*	RATE OF PROTEIN SYNTHESIS**
dna26-1	37°C	leaky START	11%	low
cdc63-1	36°C	START	30%	high
cdc63-1	40°C	NR	0.6%	very low
prt1-1	36°C	random	NR	very low
prt1-1	34°C	START	0.12%	low
prt1-1	32°C	START	17%	high
prt1-2	36°C	random	NR	low
prt1-2	34°C	START	NR	NR
prt1-3	36°C	random	NR	NR
prt1-3	34°C	leaky START	NR	NR
prt1-1564	36°C	random	NR	low
prt1-1564	34°C	leaky START	NR	NR

TABLE 5.1 Comparison of the Conditional Phenotypes Caused by Various Mutations at Different Restrictive Temperatures

The above mutants display either tight or leaky START arrest (START or leaky START respectively) or arrest at all stages of the cell cycle (random). NR - not reported. * Mating efficiency is described as the percentage of wild type mating under similar conditions. ** The rates of protein synthesis by each mutant were compared after 3hr at the respective temperatures. The data for the *dna26-1* mutant phenotype were obtained with strain TDE/16A. Other data were taken from Hanic-Joyce *et al.*, 1987a, Hartwell & McLaughlin, 1968, and Thonart *et al.*, 1976.

the *dna26-1* and *prt1* mutations would be provided by the absence of temperature resistant segregants from this cross. Complementation tests involving *dna26-1* and the available alternative alleles of *prt1* may also yield evidence of allelism. Suppression of the *dna26-1* mutation by the minimum cloned *PRT1* gene sequence carried on a centromeric plasmid would give further strong evidence of allelism.

If the *dna26-1* and *prt1* mutations prove to be definitely allelic it will be of interest to re-examine the temperature sensitive phenotype of the *dna26-1* mutant at a range of restrictive temperatures for reasons mentioned previously. It would also be of interest to examine the *dna26-1* mutant phenotype in an alternative genetic background. The contrasting mild inhibition of protein synthesis observed in a *prt1-63* START mutant at 36°C (Hanic-Joyce, *et al.*, 1987a) and more severe inhibition observed in the *dna26-1* mutant at a similar temperature suggests that the *dna26-1* mutation may cause a tighter START arrest in an alternative genetic background to that employed in the present experiments.

If however experiments indicate that the *DNA26* and *PRT1* genes are non-allelic it will be of interest to precisely map the *DNA26* locus on chromosome XV. This may be carried out by tetrad analysis, using the *dna26-1* temperature sensitive phenotype as a marker in combination with other markers from chromosome XV. It will also be necessary to verify that the cloned DNA sequence isolated in plasmid p801 does indeed contain the *PRT1* gene. This can be achieved by directing the integration of the linearized YIpDE8 construct into a *prt1* genomic locus followed by tetrad analysis, or alternatively by determining the nucleotide sequence of a portion of the cloned fragment and comparing it with that of the published *PRT1* sequence.

Complementation of the *cdc63-1* mutation by the plasmid construct YCpDE8 would also provide verification that *PRT1* has been re-cloned.

Cell Cycle Control Mediated Through Protein Synthesis

Mutations in the *PRT1* gene are able to cause a concerted cessation of cellular proliferation at START even when net protein accumulation in the cell is continuing at a high rate (Hanic-Joyce *et al.*, 1987a). This suggests that the Prt1 protein has a specific regulatory function in cell cycle control and that the attainment by cells of a minimum size at START is necessary but not sufficient for cell cycle initiation.

Experiments have been carried out to investigate the nature of the mechanism controlling the cell cycle at START, including the growth of wild type cells under limiting concentrations of amino acids and treatment of cells with the protein synthesis inhibitor cycloheximide (Shilo *et al.*, 1978b). These experiments revealed that the rate of cell cycle initiation at START is always more sensitive to external conditions than the rate of protein synthesis. It has also been demonstrated that large cells require a period of protein synthesis prior to the initiation of a new cell cycle after alpha-factor arrest (Moore, 1988). Further experiments have involved the treatment of cells with a pulse of the protein synthesis inhibitor cycloheximide after their release from a mutational arrest at START (Shilo *et al.*, 1979). This treatment caused cells to display a delay in cell cycle initiation after their release from the protein synthesis inhibitor, that was longer than the period of treatment. Protein synthesis itself showed no such delay, further suggesting the existence of one or more labile proteins required for the execution of START. Similar

cycloheximide-pulse experiments have been carried out on mammalian cells (Campisi *et al.*, 1982). These experiments revealed that oncogenically transformed mouse cells did not show a wild type delay in the G1-S transition after treatment with cycloheximide. These results suggested that the cellular transformation observed in these cells was a result of the stabilization of the labile R (restriction point) protein.

Evidence has been reported for the existence of a short-lived protein synthesized during the G1-S transition in yeast (Popolo & Alberghina, 1984). Shilo *et al.*, (1979) have suggested that cells may probe the nutrient status of the enviroment by monitoring the synthesis of specific proteins. They have suggested that instability and rapid turn-over of such regulatory proteins would allow the cell to carry out a rapid response to changes in nutrient availability in the enviroment. Thus when nutrient supply begins to become limiting the cell is prevented from initiating a new cell cycle, causing its arrest in G1 where it is least vulnerable to nutrient depletion. Extending this hypothesis has been the proposal that the Prt1 protein effects a differential effiency of translation of the mRNA's for key cell cycle regulatory proteins (Hanic-Joyce *et al.*, 1987a; 1987b; Johnston & Singer, 1990). Specifically, it has been suggested that the Prt1 protein may only permit the formation of initiation complexes for the translation of START regulatory proteins during conditions that are favourable for cell cycle initiation. The deficiency for such regulatory proteins under poor nutritional conditions would be enhanced if they were also proteolytically degraded at a fast rate. Thus, the performance of START is prevented even under conditions where net protein synthesis is still continuing

at a relatively high rate.

The G1 cyclins encoded by the three *CLN* genes of *S. cerevisiae*, and which are thought to activate the $p34^{CDC28}$ kinase at START, are attractive candidates for the unstable regulatory proteins proposed above. The C-terminal truncations observed in a number of dominant mutant *CLN* gene products, that remove putatively destabilizing PEST sequences, advance cells into S-phase and prevent proper START arrest in response to nutrient starvation and pheromone signalling (Wittenberg *et al.*, 1990; Nash *et al.*, 1988; Cross, 1988). Cyclin instability has been proved in the case of the *CLN2* gene product whose half-life *in vivo* has been shown to be approximately 15min (Wittenberg *et al.*, 1990). It has been proposed that under conditions of rapid growth, when the rate of protein synthesis is proportional to the cell volume, the net accumulation of *CLN* gene products occurs only at a particular cell volume (Richardson *et al.*, 1989). This may manifest itself as the approximate critical volume required for START (Johnston *et al.*, 1977a). Superimposed on this primary size control may be more directed regulatory mechanisms that ensure the rapid reduction of cyclin levels following exit from G1 and in response to pheromone signalling and nutrient starvation. It is already known that the regulation of the *CLN1* and *CLN2* genes in response to mating pheromone occurs at the transcriptional level (Wittenberg *et al.*, 1990), whilst *CLN3* gene function is regulated at the post-transcriptional level (Nash, *et al.*, 1988; Elion *et al.*, 1990). It is conceivable that a differential translation of G1-cyclin transcripts mediated by the *PRT1* gene product in response to enviromental signals provides a further regulatory mechanism for cell cycle control at START.

Finally, there is further evidence that arrest of cells in the G1 phase of the cell cycle may require the preferential synthesis of certain proteins including so-called G0 proteins (Iida & Yahara, 1984c). Mutants containing disruptions in the *UBI4* gene fail to arrest in G1 in response to nutrient starvation (Tanaka *et al.*, 1988). Moreover it has been shown that *cdc33* START mutants carrying a temperature labile eIF4-E mRNA cap-binding protein preferentially translate *UBI4* mRNA (Brenner *et al.*, 1988).

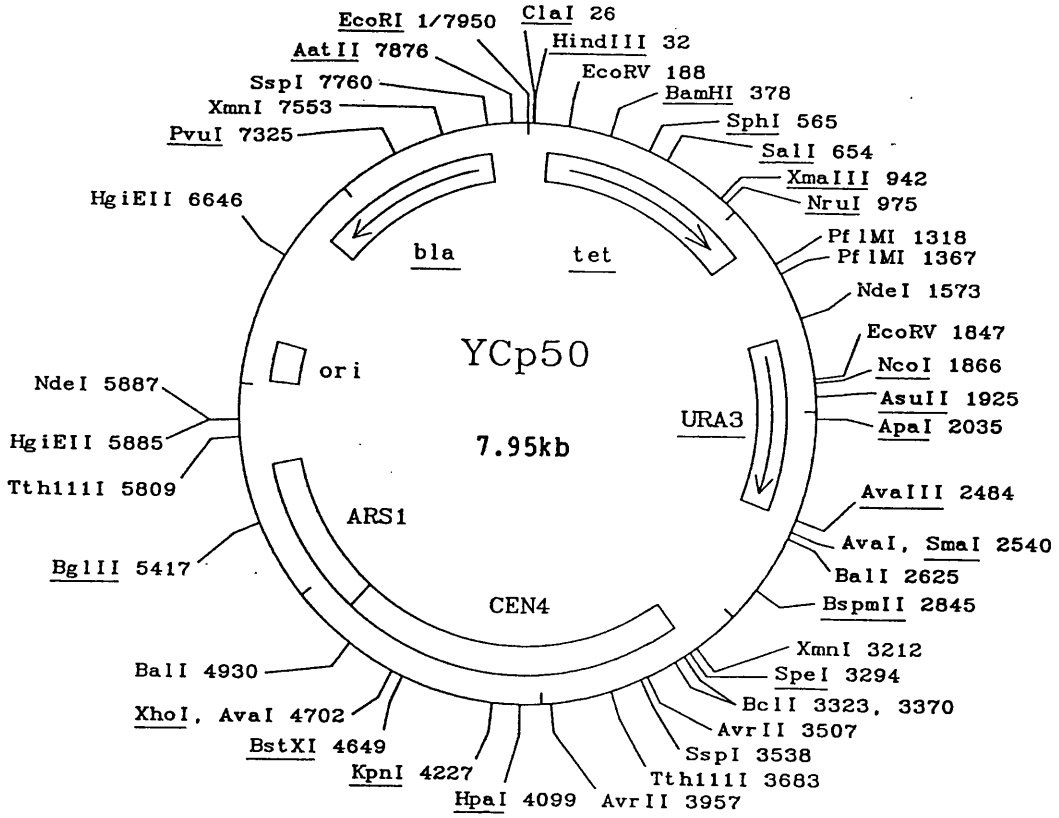
Future Work Involving the *dna26-1* Mutation

Whether or not the *dna26-1* mutation is an allele of *PRT1* it nevertheless appears to be affecting cell cycle control at START. The proposed role for the *PRT1* gene product in the differential translation of transcripts that are crucial for START regulation is therefore similarly applicable to *DNA26*, although such a role will be better substantiated for *dna26-1* if conditions can be identified under which it is able to cause a tight START arrest. Proof of this function might be obtained through experiments to identify proteins whose synthesis is specifically blocked in *prt1/dna26-1* mutants compared with wild type cells. The G1 cyclin encoded by the *CLN3* gene is a good candidate for such an analysis for two reasons apart from its apparent role in START control. Firstly, the level of the *CLN3* transcript has been shown to remain constant during the cell cycle suggesting that *CLN3* function in G1 may be regulated post-transcriptionally (Nash *et al.*, 1988; Wittenberg *et al.*, 1990). Secondly, the physical isolation of this gene has made the study of its product potentially amenable to immunoblot analysis (Nash *et al.*, 1988; Wittenberg *et al.*, 1990).

It may also be informative to investigate the effect of mutations in the nutrient or pheromone signalling pathways on the *dna26-1* mutant phenotype. For example it has been observed that the conditional G1 arrest phenotype caused by the *cdc33* START mutation is suppressed by a mutation in the *bcy1* gene encoding the regulatory sub-unit of the cAMP-dependent protein kinase (Brenner *et al.*, 1988). The latter observation suggests that nutrient-dependent phosphorylation by the cAPK is able to directly or indirectly regulate the translation of transcripts encoding key cell cycle regulatory proteins. In the light of these observations it is intriguing to recall that the Prt1 polypeptide contains two consensus motifs that represent potential sites for phosphorylation by the cAPK (Hanic-Joyce *et al.*, 1987b).

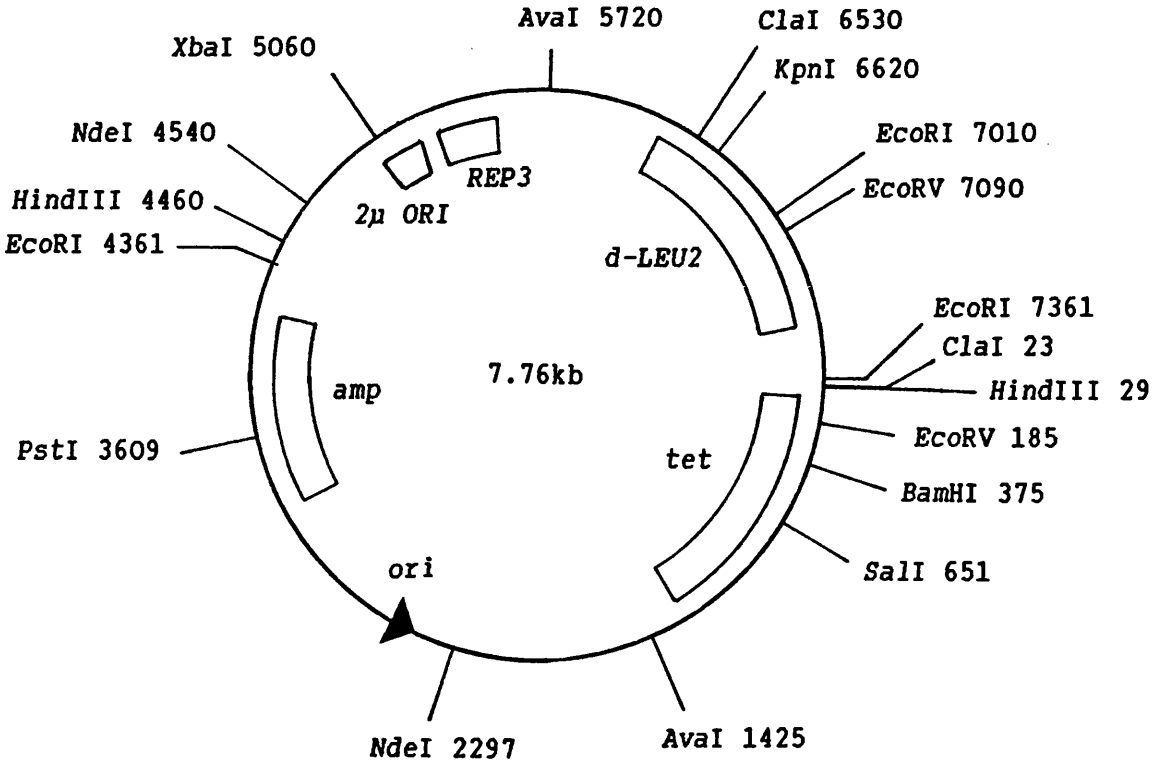
Studies that are presently being carried out on the *dna26-1* mutation include an investigation of its effect on nutrient-induced protein phosphorylation (Johan Thevelein, personal communication). Current study of the *PRT1* gene includes analysis of the differential translation carried out by the wild type and mutant gene products under permissive and restrictive conditions and under conditions of heat shock (Richard Singer, personal communication).

APPENDIX 1 : VECTOR RESTRICTION MAPS



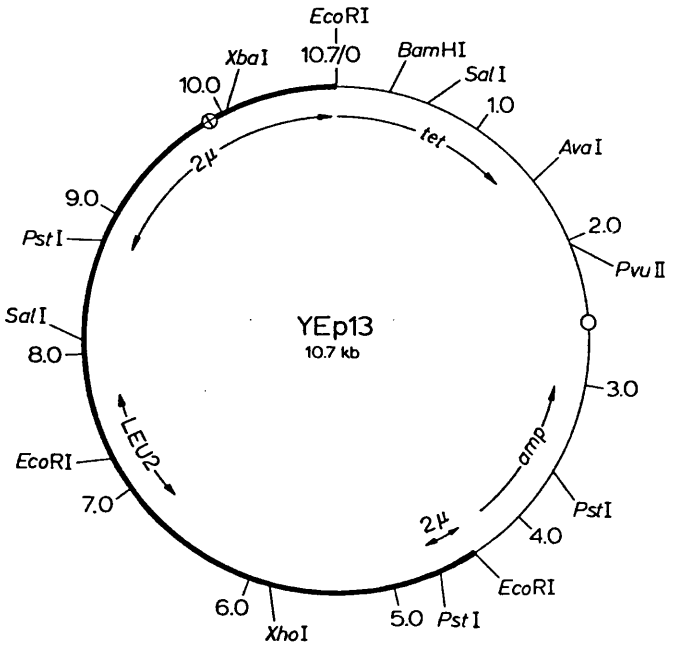
A. Restriction Map of YCp50

YCp50 was used in the present study for sub-cloning procedures and a gene library derivative was used in an attempt to isolate the wild type *DNA26* gene. The map shows the restriction sites for enzymes that cut the plasmid once or twice. The numbering system indicates the first bp of the recognition sequence in all cases. The positions of the vector-borne genes are marked by boxes and the direction of transcription of the *bla*, *tet* and *URA3* genes is shown. *bla*, β-lactamase gene; *tet*, tetracycline resistance gene; *ori*, pBR322 origin of replication; *URA3*, yeast gene encoding orotidine-5'-phosphate carboxy-lyase; *CEN4* and *ARS1*, yeast centromeric and autonomous replication sequences respectively. Map taken from Rose *et al.*, (1987). YCp50 was a gift from Dick Dickinson and the library derivative was a gift from Dave Carruthers, Dalhousie University.



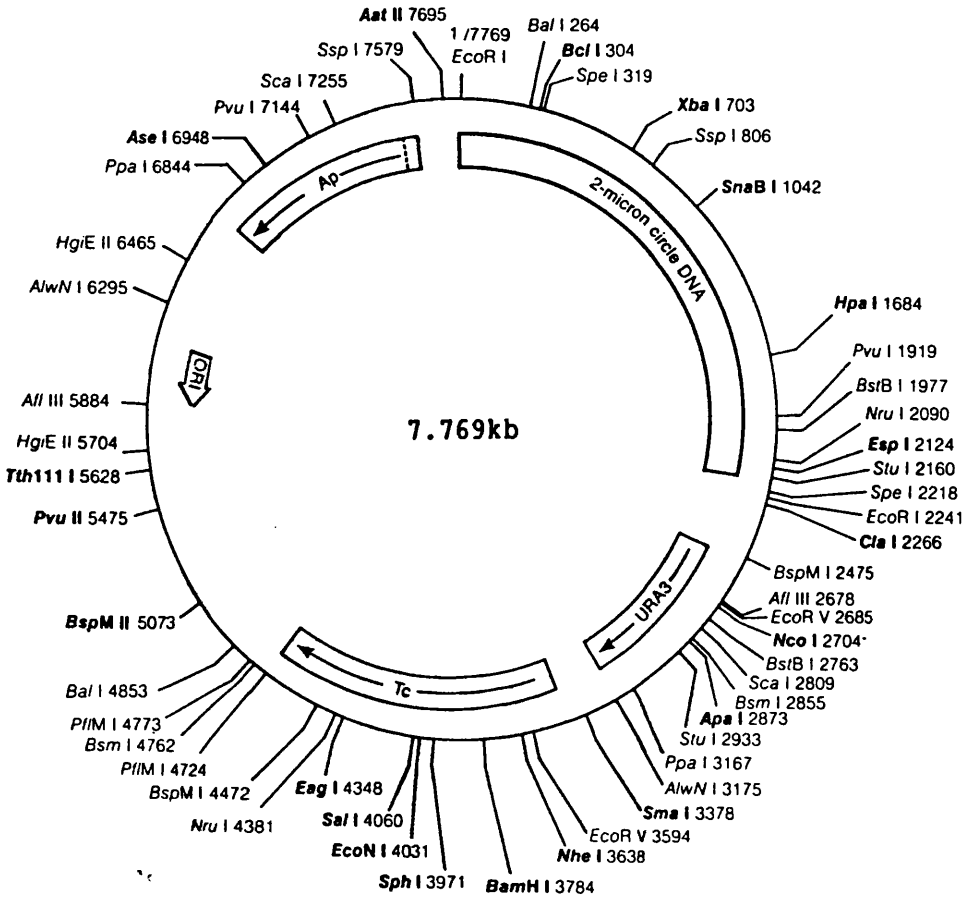
B. Restriction Map of pMA3A

A yeast gene library constructed in this vector was used to isolate a wild type gene capable of functionally complementing the *dna26-1* mutation. The vector was constructed by insertion of the 3.4kb yeast DNA cassette into the *Eco*RI site of pBR322. The gene library was constructed by inserting 10-15kb partial *Sau*3A genomic fragments from a wild type yeast strain into the unique *Bam*HI site of pMA3A (Mick Tuite, personal communication). Coordinates of restriction sites in the bacterial sequences were taken from the pBR322 map (Appendix 2(F)). *amp*, beta-lactamase (ampicillin resistance) gene; *tet*, tetracycline resistance gene; ▶ *ori*, *ColE1* origin of replication; 2μ *ORI*, 2μm plasmid origin of replication; *REP3*, 2μm plasmid *REP3* gene; *d-LEU2*, yeast "defective" *LEU2* gene encoding isopropylmalate dehydrogenase (Erhart & Hollenberg, 1983). Both pMA3A and the library derivative were a gift from Mick Tuite, Kent University.



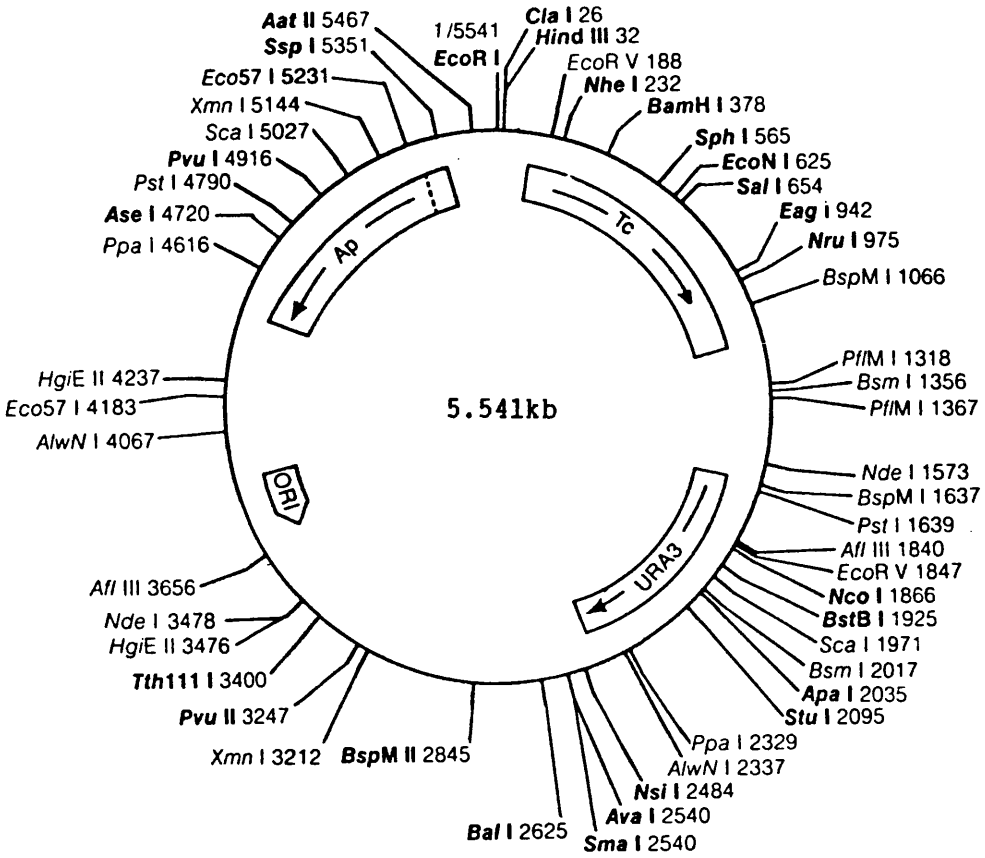
C. Restriction Map of Plasmid YEp13

YEp13 was used in the present study as a control plasmid during the development of an efficient yeast transformation protocol. Thin line - pBR322 sequences; thick line - yeast DNA sequences. The approximate locations of the various vector-borne genes are indicated by arrows. The cleavage sites for a range of restriction endonucleases are also shown. *tet*, tetracycline resistance gene; *amp*, ampicillin resistance gene; *LEU2*, yeast gene encoding isopropylmalate dehydrogenase contained on a 4.1kb *Pst*I fragment and inserted into a 2.2kb *Eco*RI fragment containing sequences from the B-Form of the yeast 2μm plasmid. Open circle, pBR322 origin of replication; crossed circle, 2μm origin of replication. Map taken from Pouwels *et al.*, 1985.



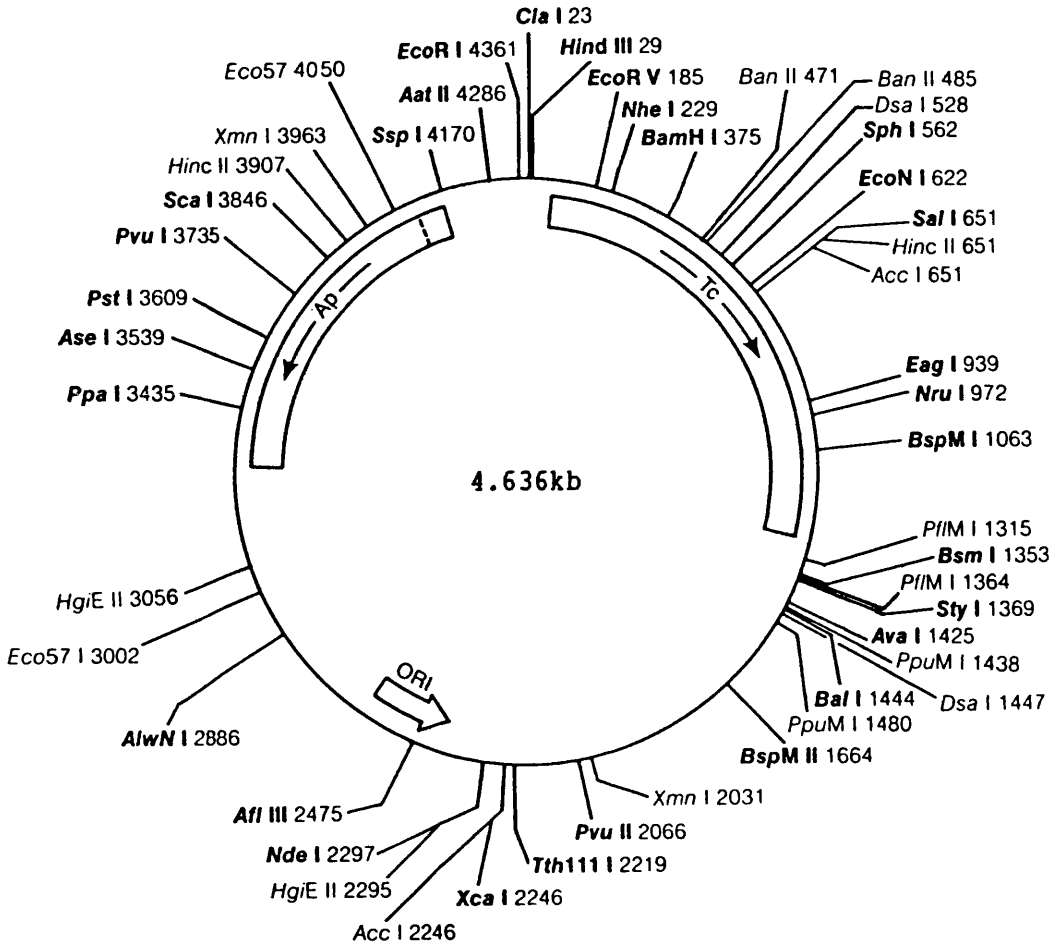
D. Restriction Map of YEp24

YEp24 was used in this study as a vehicle for the sub-cloning of a genomic yeast DNA fragment capable of functionally complementing the *dna26-1* mutation. The numbering of the nucleotide sequence in the above map begins at the first G in the *Eco*RI site at the junction of the pBR322 and 2µm circle sequences. Restriction sites are shown for those enzymes that cut the molecule once or twice, with the coordinates of each refering to the first 5' base in the recognition sequence. The map also shows the location and direction of transcription of the various genes. Ap, ampicillin resistance gene; Tc, tetracycline resistance gene; ORI, pBR322 origin of replication; *URA3*, yeast gene encoding orotidine-5'-phosphate carboxy-lyase. Map taken from New England BioLabs Inc., 1988-89 catalogue.



E. Restriction Map of YIp5

YIp5 was used in the present study as a vehicle for the integration of a cloned DNA fragment into the genome of the *dna26-1* mutant TDE/16A prior to linkage mapping. The numbering of the nucleotide sequence shown in the above map begins at the G of the pBR322-derived GAATTC *Eco*RI recognition site. The sites for enzymes that cut the molecule once or twice are shown, with the coordinates of each refering to the first 5' base in the recognition sequence. The map also shows the location and direction of transcription of the various genes. Ap, ampicillin resistance gene; Tc, tetracycline resistance gene; ORI, pBR322 origin of replication; *URA3*, yeast gene encoding orotidine-5'-phosphate carboxy-lyase. The plasmid was a gift from Finton Walton. Map taken from New England BioLabs Inc., 1988-89 catalogue.



F. Restriction Map of pBR322

The base pairs of the pBR322 genome are numbered arbitrarily from the centre of the unique *Eco*RI site. Nucleotide position +1 is therefore the first T in the *Eco*RI GAATTC recognition sequence. The coordinate assigned to each cleavage site in the map represents the base immediately 5' to the particular enzyme-catalyzed nick in the clockwise 5'-3' strand. The map shows the restriction sites of those enzymes that cut the molecule once or twice. Ap, ampicillin resistance gene; Tc, tetracycline resistance gene; ORI, pMB1 origin of replication (derived from ColEI). Data from Old & Primrose, (1985), and map taken from New England BioLabs, 1988-89 catalogue.

APPENDIX 2 : RESTRICTION MAPPING DATA FOR PLASMID p801

A. Restriction Data For Plasmid p801

RESTRICTION ENZYME	SIZE OF RESTRICTION FRAGMENTS (kb)
*Bam*HI	> 11.0
*Xba*I	8.3, 5.6
*Eco*RV	14.5, 0.86
*Pst*I	10.1, 5.2
*Sal*I	12.7, 1.65
*Ava*I	9.8, 3.9
*Nde*I	11.0, 2.4
*Cla*I	12.2, 3.15, 1.25
*Bgl*II	11.4, 1.47, 0.48
*Eco*RI	4.53, 4.18, 2.65, 1.70, 0.76, 0.57, 0.43, 0.32

The data are for the size of the restriction fragments generated by digestion of plasmid p801 with a range of restriction endonucleases.

B. Data From Double Restriction Digestion of Plasmid p801

RESTRICTION ENZYMES	SIZE OF RESTRICTION FRAGMENTS (kb)
EcoRI/HindIII	3.8, 2.8, 2.65, 1.7, 1.4 0.79, 0.59, 0.41, 0.35 0.11, 0.09
EcoRI/KpnI	4.53, 2.95, 2.45, 1.74, 1.26, 0.81, 0.59, 0.40, 0.33, 0.26, 0.24
EcoRI/SalI	3.8, 2.75, 2.75, 1.85, 1.31, 0.58, 0.81, 0.54, 0.39, 0.34
EcoRI/EcoRV	4.38, 3.96, 2.84, 1.54, 1.71, 0.61, 0.42, 0.35, 0.26, 0.2
EcoRV/SalI	6.2, 5.2, 1.77, 0.84
EcoRV/PstI	10.0, 3.5, 0.86, 0.59
EcoRV/ClaI	11.4, 3.15, 0.69, 0.58, 0.26
AvaI/KpnI	4.35, 4.0, 3.6, 1.7, 0.88
AvaI/HindIII	4.4, 3.65, 2.8, 2.03, 1.28
AvaI/ClaI	4.8, 4.2, 3.25, 1.27, 0.8
EcoRI/BglII	4.25, 2.75, 1.9, 1.75, 1.56, 0.80, 0.58, 0.45, 0.39, 0.33
EcoRI/AvaI	4.3, 3.15, 1.69, 1.37, 1.27, 0.78, 0.56, 0.38, 0.32
EcoRI/BamHI	4.1, 3.8, 2.85, 1.38, 0.81, 0.59, 0.4, 0.4, 0.34
EcoRI/ClaI	4.55, 3.45, 1.7, 2.33, 0.8, 5.9, 5.9, 0.49, 0.4, 0.34
EcoRI/XbaI	4.13, 3.96, 2.1, 1.65, 0.8 0.73, 0.6, 0.36, 0.28, 0.23
EcoRI/PstI	4.1, 3.45, 2.9, 0.97, 0.79 0.77, 0.58, 0.40, 0.35
EcoRV/BglII	6.65, 4.93, 1.67, 0.88, 0.46
EcoRV/XbaI	11.0, 2.25, 2.07, 0.85
AvaI/BglII	7.4, 5.1, 1.55, 1.34, 0.43
AvaI/SalI	7.35, 4.5, 1.85, 0.8
AvaI/PstI	7.8, 2.55, 2.0, 1.95

B. (Cont.). Data from Double Restriction Digestion of Plasmid p801

RESTRICTION ENZYMES	SIZE OF RESTRICTION FRAGMENTS (kb)	RESTRICTION ENZYMES	SIZE OF RESTRICTION FRAGMENTS (kb)
BglII/HindIII	4.3, 3.51, 1.5, 0.53, 0.47	*BglII/NdeI*	8.0, 2.5, 2.05, 1.53, 0.48
BglII/ClaI	6.1, 3.71, 1.42, 1.57, 1.3, 0.46	*BglII/PstI*	5.15, 4.43, 3.78, 1.53, 0.46
BglII/XbaI	5.4, 5.15, 1.84, 1.53, 0.46	*ClaI/NdeI*	5.0, 3.45, 2.3, 1.8, 1.3
ClaI/KpnI	8.7, 2.14, 1.18, 1.08, 0.61	*ClaI/HindIII*	6.3, 3.7, 2.45, 1.31, 0.78
ClaI/PstI	7.2, 2.89, 2.68, 1.3, 0.75	*ClaI/XbaI*	9.6, 2.41, 1.5, 1.28, 1.04
PstI/KpnI	6.05, 2.68, 1.78, 1.64, 1.58	*PstI/HindIII*	5.5, 3.75, 3.53, 0.88, 0.77
PstI/XbaI	8.0, 3.43, 1.7, 1.45	*XbaI/SalI*	5.4, 4.1, 3.18, 1.82
XbaI/NdeI	6.2, 5.0, 2.2, 0.4	*XbaI/KpnI*	6.8, 3.43, 1.61, 1.55
XbaI/HindIII	6.5, 2.82, 2.5, 1.9, 0.74	*HindIII/NdeI*	4.65, 4.25, 3.3, 2.0, 0.29
HindIII/KpnI	6.4, 2.15, 1.99, 1.65, 1.2		

The data are for the size of restriction fragments generated by simultaneous digestion of p801 DNA by two restriction endonucleases.

C. Data from Triple Restriction Digestion of Plasmid p801

RESTRICTION ENZYMES	SIZE OF RESTRICTION FRAGMENTS (kb)
*Kpn*I/*Ava*I/*Pst*I	3.9, 2.25, 2.2, 2.0, 1.77, 1.73, 0.88
*Ava*I/*Bgl*II/*Cla*I	5.2, 4.15, 3.2, 1.46, 1.35, 1.28, 1.23, 0.78, 0.43
*Pst*I/*Bgl*II/*Cla*I	3.37, 2.79, 2.41, 1.54, 1.45, 1.33, 0.77, 0.48
*Pst*I/*Bgl*II/*Xba*I	3.48, 3.15, 1.72, 2.27, 1.53, 1.47, 0.48
*Pst*I/*Bgl*II/*Eco*RV	4.09, 3.58, 3.15, 1.51, 0.87, 0.61, 0.48
*Xba*I/*Cla*I/*Bgl*II	4.84, 2.34, 1.52, 1.45, 1.45, 1.30, 1.07
*Xba*I/*Eco*RV/*Bgl*II	4.68, 2.3, 2.18, 2.02, 1.52, 0.89, 0.48
*Cla*I/*Eco*RV/*Bgl*II	6.30, 3.15, 1.57, 1.45, 0.74, 0.65, 0.48
*Pst*I/*Xba*I/*Eco*RV	5.95, 2.08, 1.7, 1.4, 0.72, 0.48
*Xba*I/*Pst*I/*Cla*I	5.55, 1.74, 1.46, 1.42, 1.39, 0.95, 0.59
*Eco*RV/*Cla*I/*Xba*I	6.65, 2.2, 1.43, 0.93, 0.57, 0.5, 0.15

The data are for the size of restriction fragments generated by the simultaneous digestion of p801 DNA by three restriction endonucleases.

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